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## Role of Decidual Corticosteroid production in Reproductive Failure

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Submitted to the University of Warwick  
Faculty of Reproductive Medicine  
For the Degree of Doctor of Medicine

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# Table of Contents

<b>Declaration and Acknowledgement .....</b>	<b>5</b>
<b>Acknowledgements .....</b>	<b>6</b>
<b>Abstract .....</b>	<b>7</b>
<b>List of Abbreviations .....</b>	<b>9</b>
<b>List of Figures and Tables.....</b>	<b>13</b>
<b>1.0 INTRODUCTION .....</b>	<b>16</b>
1.1    The Human Endometrium.....	16
1.1.1    Structure of the endometrium.....	17
1.1.2    Cyclical changes of the human endometrium .....	19
1.2    Menstruation .....	22
1.2.1    Purpose of menstruation .....	23
1.3    Decidualization.....	24
1.3.1    Functions of the decidua.....	26
1.3.2    Progesterone and cAMP signalling Pathways.....	27
1.4    Endometrial receptivity and window of implantation.....	28
1.5    Implantation.....	32
1.5.1    Pre-implantation embryo development .....	32
1.5.2    Implantation process .....	34
1.6    Immunomodulation .....	35
1.6.1    Immune cells in the endometrium.....	35
1.6.2    Peripheral Natural Killer (NK) cells.....	36
1.6.3    Human uterine Natural Killer (uNK) cells .....	36
1.6.4    HLA compatibility .....	45
1.6.5    CD3+ T cells .....	45
1.6.6    Macrophages.....	46
1.6.7    Cytokines.....	46
1.6.8    Th1/Th2 balance in pregnancy.....	48
1.7    Steroid hormone regulation of endometrium .....	48
1.7.1    Steroid receptor expression in the endometrium .....	49
1.7.2    Glucocorticoids .....	50
1.8    Clinical Perspective .....	53

1.8.1	Reproductive failure.....	54
1.8.2	Infertility.....	55
1.8.3	Recurrent Implantation Failure (RIF) .....	55
1.8.4	Recurrent miscarriage.....	56
1.8.5	Endometrium in RM.....	57
1.8.6	Glucocorticoids in RM .....	58
1.9	Hypothesis and Aims.....	61
<b>2.0</b>	<b>Materials and Methods .....</b>	<b>63</b>
2.1	Materials .....	63
2.1.1	Antibodies .....	63
2.1.2	Cells .....	64
2.1.3	Small interfering RNA (SiRNA).....	64
2.1.4	Culture media and materials.....	64
2.1.5	Reagents.....	65
2.1.6	Buffers and solutions .....	65
2.1.7	Immunohistochemistry .....	69
2.1.8	Enzyme Linked Immunoassay (ELISA) .....	69
2.2	Methods.....	71
2.2.1	Ethics Approval and Patient Recruitment.....	71
2.2.2	Timed Endometrial samples.....	71
2.2.3	Endometrial samples for primary cell culture.....	74
2.3	Cell Culture.....	75
2.3.1	General maintenance of cell cultures .....	75
2.3.2	Preparation of Dextran coated charcoal (DCC) stripped fetal calf serum (FBS) 75	
2.3.3	Primary human endometrial stromal cell culture.....	76
2.3.4	Hormone treatment of cells.....	77
2.3.5	Transient transfection.....	78
2.4	Tissue specimens .....	78
2.4.1	Protein Analysis.....	79
2.4.2	RNA isolation and extraction .....	83
2.4.3	Gene Expression Analysis using Real Time Quantitative PCR (qRT-PCR) .....	84
2.4.4	Isolation of genomic DNA and cDNA synthesis.....	86
2.4.5	Amplification of Target cDNA .....	87

2.5	Microarray analysis .....	88
2.6	Enzyme-linked immunosorbent assay (ELISA) .....	89
2.7	Confocal immunofluorescence microscopy.....	90
2.8	Immunohistochemistry (IHC).....	90
2.8.1	Principle .....	91
2.8.2	Procedure.....	91
2.8.3	Construction of Tissue Micro Array (TMA) .....	93
2.8.4	Image J Analysis .....	94
2.9	Radio-thin layer chromatography .....	97
2.10	Statistical analysis .....	98
<b>3.0</b>	<b>Human Endometrial Stromal Cells</b> .....	<b>100</b>
3.1	Introduction .....	100
3.2	Results.....	103
3.2.1	Expression patterns of 11 $\beta$ HSD1 and 2, GR, and MR in undifferentiated and decidualized HESCs & impact of progesterone and dexamethasone on this signalling pathway .....	103
3.2.2	P4 drives the expression of 11 $\beta$ HSD1 in decidualizing HESCs .....	107
3.2.3	Identification of GR and MR dependent genes in decidualizing HESCs.....	112
3.2.4	GR limits the expression Krüppel-associated box domain containing ZNF (KRAB-ZNF) transcriptional repressors .....	116
3.2.5	MR is essential for retinoid metabolism and lipid droplet biogenesis .....	118
3.3	DISCUSSION.....	121
<b>Chapter 4</b>	<b>Elevated peri-implantation uterine natural killer cell density in human endometrium is associated with impaired decidualization stromal cells in recurrent miscarriage and recurrent implantation failure patients .....</b>	<b>127</b>
4.1	INTRODUCTION.....	128
4.2	RESULTS.....	131
4.2.1	Elevated uNK cell density in vivo is associated with impaired induction of key decidual markers in vitro .....	131
4.2.1	Cytokines IL-11 and IL-15 .....	133
4.3	DISCUSSION.....	134
<b>Chapter 5:</b>	<b>Elevated peri-implantation uterine natural killer cell density in human endometrium is associated with impaired corticosteroid signalling in decidualizing stromal cells .....</b>	<b>136</b>

5.1 INTRODUCTION.....	137
5.2 RESULTS.....	138
5.2.1 Mid-luteal uNK cell density correlates inversely with endometrial 11 $\beta$ HSD1 expression.....	138
5.2.1 Elevated uNK cell density in vivo is associated with impaired expression of MR- dependent metabolic enzymes.....	142
<b>Chapter 6: Conclusion.....</b>	<b>152</b>
<b>References.....</b>	<b>160</b>
<b>Appendix .....</b>	<b>197</b>

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# Declaration and Acknowledgement

I Dr Radha Viriyur Venkatakrisnan declare that:

1. My research has been conducted ethically and all the work presented in this thesis, except where specifically stated, and was original research performed by myself under the supervision of Professor Siobhan Quenby and mentor Professor Jan Brosens.
2. I was part of large team that did this work. My role was to recruit patients into my study and perform tissue biopsies. I have actively helped with the cell culture work and laboratory procedures under the able supervision of our scientist Keiji Kuroda and senior biomedical scientist Sean James.
3. The data and results presented are genuine data and results obtained during the conduct of my research.
4. I have appropriately acknowledged and referred within my thesis, where I have drawn on the work, ideas and help of others.
5. The thesis submitted is within the required word limit as specified by the University of Warwick.

# Acknowledgements

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I wish to thank my son Karthik and my husband Anil who have understood and strongly supported through the ups and downs during this period.



## **Abstract**

Glucocorticoids have been implicated in many processes including successful embryo implantation, placentation, and the growth and development of the fetus. Glucocorticoid treatment has been advocated as a treatment to improve reproductive outcome for a number of reasons. Prednisolone treatment has been associated with improvement in clinical outcome in women with recurrent miscarriage and improvement in outcome of In Vitro Fertilisation. Steroids have been found to reduce the high levels of uterine natural killer cells which have been associated with recurrent miscarriage and recurrent implantation failure. Glucocorticoids stimulate peri-implantation human chorionic gonadotrophin secretion from trophoblast of early human embryo and accelerate trophoblast growth and invasion. Elevated uterine NK cell levels during the implantation window are associated with reproductive failure and can be repressed by oral glucocorticoids.

We have shown that decidualizing human endometrial stromal cells profoundly up regulate the expression and activity of 11beta-hydroxysteroid dehydrogenase type 1, the enzyme that converts inert cortisone to active cortisol; thus establishing a local cortisol gradient and activation of glucocorticoid and mineralocorticoid receptors. We also found that elevated levels of uterine natural killer cells in the stroma underlying the surface epithelium of endometrium are associated with defective decidualization of resident stromal cells, inadequate cortisol biosynthesis and suboptimal

induction of corticosteroid-dependent enzymes involved in lipid droplet accumulation and retinoid transport pathway.

Thus, impaired decidualization limits the induction of a local cortisol gradient in the stroma underlying the surface epithelium. This in turn accounts for possible inappropriate recruitment of uterine natural killer cells and suboptimal expression of metabolic genes involved in lipid biosynthesis and retinoid storage pathway.

Based on the findings, we postulate that patients suffering recurrent miscarriage associated with high uterine NK cell density may benefit from corticosteroid treatment in early pregnancy; although this assumption will need to be tested in a larger clinical trial.

# List of Abbreviations

%	Percent
<sup>0</sup> C	Centigrade
8-Br-cAMP	8-Bromoadenosine-3',5'-cyclic monophosphate
11 $\beta$ HSD1	11 $\beta$ Hydroxysteroid dehydrogenase 1
11 $\beta$ HSD2	11 $\beta$ Hydroxysteroid dehydrogenase 2
A.U	Arbitrary Units
ACTH	Adrenocorticotrophic hormone
AF	Activation function
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP1	Activating protein 1
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
C/EBP $\beta$	CCAAT/enhancer binding protein $\beta$
Ca <sup>2+</sup>	Calcium ions
CaCl <sub>2</sub>	Calcium Chloride
cAMP	Cyclic Adenosine Monophosphate
CaPO <sub>4</sub>	Calcium phosphate
CBX	Carbenoxolone
CD3,4,8	Cluster of Differentiation molecules 3,4,8
CD56 bright	Cluster of Differentiation molecule 56 bright
cDNA	Complementary DNA
Cl <sup>-</sup>	Chloride ions
cm <sup>2</sup>	Centimeter squared
CO <sub>2</sub>	Carbon dioxide
CRE	cAMP response elements
CREB	cAMP response element binding proteins
CREM	cAMP response element modulator
CRH	Corticotrophin-releasing hormone
C <sub>t</sub>	Threshold cycle
d	Days
DAB	3,3'-diaminobenzidine
Dap1p	Damage associated reponse protein
DAP1	4'-6-diaminodino-2phenylindole
DBD	DNA-binding domain
DBS	DNA binding site
DCC	Dextran coated charcoal
DCF	2',7'-dichlorofluroscein
DCFH-DA	2',7'-dichlorofluroscein-diacetate
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone
dH <sub>2</sub> O	Distilled water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid

DNase1	Deoxyribonuclease 1
E	Cortisone
E <sub>2</sub>	Estradiol
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immunosorbent Assay
ER	Estrogen receptor
ERβ1&2	Estrogen Receptors β 1&2
F	Cortisol
FBS	Fetal bovine serum
FSH	Follicle Stimulating Hormone
g	Gram
<i>g</i>	Gravity force
GnRH	Gonadotrophin releasing hormone
GPCR	G-protein coupled receptor
GPx	Glutathione peroxidase
GR	Glucocorticoid Receptor
h	Hour
H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>2</sub>	Hydrogen Peroxide
HBSS	Hank's balanced salt solution
hCG	Human Chorionic gonadotropin
HCl	Hydrochloric acid
HEPES	4-(2-Hydroxyethyl)-1piperazineethanesulfonic acid
HESCs	Human endometrial stromal cells
HRP	Horseradish peroxidase
IF	Immunofluorescence
IGFBP1	Insulin- like growth factor binding protein-1
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IP3	Inositol-1,4,5-triphosphate
IVF	In Vitro Fertilization
KCl	Potassium Chloride
kDa	Kilodaltons
L	Litre
L-Cor	Ligand-dependent nuclear receptor co-repressor
LBD	Ligand binding domain
LH	Luteinizing Hormone
M	Molar
mg	Milligram
MgCl <sub>2</sub>	Magnesium Chloride
Min	minute
MMPs	Matrix Metalloproteinases
MPA	17α- medroxyprogesterone acetate
MR	Mineralocorticoid receptor
m-RNA	Messenger RNA
NA	Nicotinic acid
NaCl	Sodium Chloride
NADPH	Nicotinamide dinucleotide phosphate
NK	Natural Killer cells

nM	NanoMolar
nm	Nanometer
NP-40	Nonidet P-40
NSB	Non-specific Binding protein
NT	Non targeting
O/N	Over night
O <sub>2</sub> <sup>-</sup>	Superoxide anion
O <sub>2</sub>	Molecular Oxygen
P4	Progesterone
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
PKA	Protein Kinase A
PKG	Protein Kinase G
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
PRL	Prolactin
PVDF	Polyvinylidene fluoride
RETSAT	Retinol Saturase
RIF	Recurrent Implantation Failure
RIPA	RadiolImmuno precipitation assay
RLU	Relative lightunits
RLX	Relaxin
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPM	Revolution per minute
RT-PCR	Reverse transcriptase –PCR
RTP	Room temperature and pressure
RTQ-PCR(qRT PCR)	Real Time quantative Polymerase Chain Reaction
SB	Specific Binding
SD	Standard Deviation
SDS	Sodium dodecylsulphate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
SEM	Standard error of mean
siRNA	small interfering RNA
SOD2	Superoxide dismutase 2
SRC	Steroid receptor co-activator
STAT	Signal Transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
TB	total binding
TBE	Tris-borate EDTA
TBS	Tris Buffered Saline
TBS-T	TBS-Tween
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TF	Tissue factor
TGFβ	Transforming Growth factor β
TMA	Tissue microarray
TNF	Tumour necrosis factor

Tris	Trishydroxymethylaminomethane
TXN	Thioredoxin
U	Units
uNK	Uterine Natural Killer cells
V	Volts
v/v	Volume/Volume
w/v	Weight/Volume
WB	Western Blot
Wt	Wild Type
μg	Microgram

# List of Figures and Tables

<b>FIGURE 1.1:</b> AN ILLUSTRATION OF HUMAN ENDOMETRIUM.....	17
<b>FIGURE 1.2:</b> THE UTERINE BLOOD SUPPLY.....	19
<b>FIGURE 1.3:</b> ENDOMETRIAL CYCLE UNDER THE INFLUENCE OF OESTROGEN AND PROGESTERONE IN PROLIFERATIVE AND SECRETORY PHASE RESPECTIVELY.....	22
<b>FIGURE 1.4:</b> ENDOMETRIUM IN MENSTRUAL CYCLE SHOWING THE 'WINDOW OF IMPLANTATION OR RECEPTIVE PERIOD.....	31
<b>FIGURE 1.5:</b> THE 11BHSD ISOENZYMES: 11BHSD1 IS A NADPH-DEPENDENT REDUCTASE WHICH CONVERTS INACTIVE CORTISONE TO ACTIVE CORTISOL AND 11BHSD2 IS A NAD-DEPENDENT DEHYDROGENASE ENZYME REVERSES THE CONVERSION FROM ACTIVE CORTISOL TO INACTIVE CORTISONE.....	52
<b>FIGURE 1.6:</b> OVERVIEW OF OUTCOME OF 100 CONCEPTIONS.....	54
<b>FIGURE 1.7:</b> SCHEMATIC REPRESENTATION SHOWING GLUCOCORTICOIDS ACTING ON THE UNK CELLS VIA THE GC RECEPTORS.....	59
<b>FIGURE 2.1:</b> SCHEMATIC PROCESS FROM TISSUE SAMPLING TO ANALYSIS.....	90
<b>FIGURE 3.1:</b> EXPRESSION OF 11BHSD ENZYMES, GR, AND MR IN DECIDUALIZING HESC.....	103
<b>FIGURE 3.2</b> SWITCH FROM GR TO MR DOMINANCE UPON DECIDUALISATION OF HESC'S.....	104
<b>FIGURE 3.3</b> THE IMPACT OF P4 AND DEX TREATMENT ON 11BHSD1 EXPRESSION UPON DECIDUALIZATION.....	106
<b>FIGURE 3.4:</b> EFFECT OF CORTISONE (E) ON THE EXPRESSION OF DECIDUAL MARKER GENES. PRIMARY HESCS WERE DIFFERENTIATED WITH 8-BROMO-CAMP AND P4 IN THE PRESENCE OR ABSENCE OF E.....	107
<b>FIGURE 3.5:</b> EFFECT OF 11BHSD1 INHIBITION ON THE EXPRESSION OF DECIDUAL MARKER GENES AND GR/MR-DEPENDENT GENES.....	108
<b>FIGURE 3.6:</b> IDENTIFICATION OF GR- AND MR-REGULATED GENES IN DECIDUALIZING HESCS.....	111
<b>FIGURE 3.7:</b> EXPRESSION AND VALIDATION OF PUTATIVE GR- AND MR-DEPENDENT GENES IN DECIDUALIZING HESCS.....	112

<b>FIGURE 3.8:</b> GR SIGNALLING PLAYS A ROLE IN CHROMATIN REMODELLING IN DECIDUALIZING HESCS. ....	114
<b>FIGURE 3.9:</b> MR-DEPENDENT INDUCTION OF DHRS3 IN DECIDUALIZING HESCS IS ASSOCIATED WITH DYNAMIC CHANGES IN CYTOPLASMIC LIPID DROPLETS.....	117
<b>FIGURE 4.1:</b> INVERSE CORRELATION BETWEEN UNK CELL DENSITIES IN VIVO AND THE INDUCTION OF DECIDUAL MARKERS IN VITRO IS NOTED.....	129
<b>FIGURE 4.2:</b> INDUCTION OF IL11 AND IL15 MRNA IN DECIDUALIZING HESCS IN CULTURE DOES NOT CORRELATE TO UNK CELL DENSITIES IN VIVO.....	130
<b>FIGURE 5.1:</b> INVERSE CORRELATION BETWEEN UNK CELL DENSITY IN THE SUBLUMINAL EPITHELIUM AND EXPRESSION OF 11BHSD1 IS NOTED.....	136
<b>FIGURE 5.2:</b> THE EXPRESSION PATTERNS OF 11BHSD1, PRL AND IGFBP1 IN DECIDUALIZING ENDOMETRIAL STROMAL CELLS AND THEIR CORRELATION TO THE UNK CELL POPULATIONS.....	137
<b>FIGURE 5.3:</b> IMMUNOHISTOCHEMISTRY - GR AND MR STAINING OF UNDIFFERENTIATED HESCS.....	139
<b>FIGURE 5.4:</b> THE EXPRESSION OF DHRS3 AND RETSAT UPON DECIDUALIZATION.....	140
<b>FIGURE 5.5:</b> 11BHSD1, DHRS3 AND RETSAT PROTEIN EXPRESSION IN DECIDUALIZING ENDOMETRIAL CELLS DEPENDING ON UNK CELL COUNT.....	141
 <b>TABLE 1:</b> COMPARISON OF CLINICAL AND ENDOMETRIAL CHARACTERISTICS OF WOMEN IN THE STUDY- CONTROLS, RMC WITH LOW UNK CELL POPULATION AND RMC WITH HIGH UNK CELL POPULATION.....	 69
 <b>TABLE 2:</b> PATIENT CHARACTERISTICS OF WOMEN IN THE CELL CULTURE EXPERIMENTS- CONTROLS, RMC WITH LOW UNK CELL POPULATION AND RMC WITH HIGH UNK CELL POPULATION.....	 70

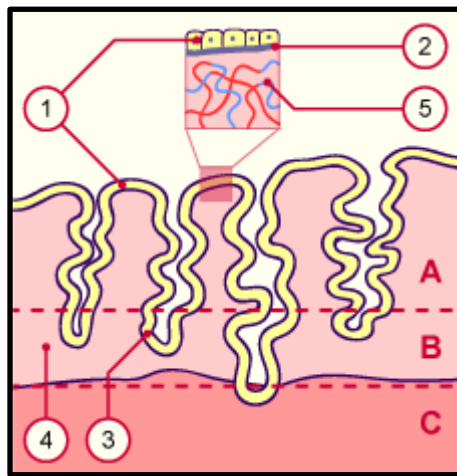


# **Chapter 1: Introduction**

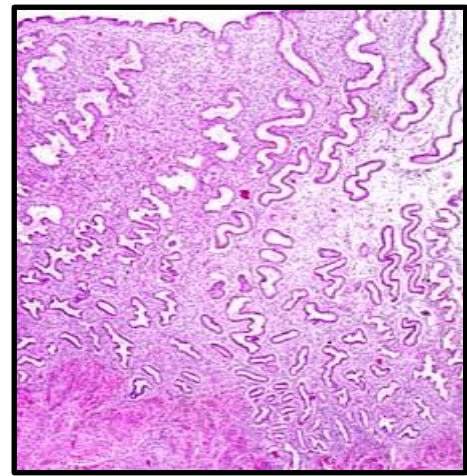
# 1.0 INTRODUCTION

## 1.1 The Human Endometrium

The endometrium is the innermost glandular layer and functions as a lining for the uterus. The endometrium is a dynamic tissue which, in response to the cyclical changes in levels of oestrogen and progesterone, undergoes recurrent proliferation, differentiation and tissue breakdown (Jabbour *et al.*, 2006; Salamonsen, 2003). The key role of the human endometrium is to orchestrate the events that lead to fertilization, implantation and pregnancy. The endometrium is composed of the basal and functional layers (Fig 1.1). The basal layer is the deep layer adjacent to the myometrium, while the functional layer is the superficial two-thirds of the endometrium. The functional layer is again divided into stratum compactum and stratum spongiosum. Stratum compactum is the superficial thin layer with gland necks and dense stromal cells while the deeper stratum spongiosum is composed of glands and loosely arranged stroma. The functional layer of endometrium is shed each menstrual cycle (Nair & Taylor, 2010). The basal layer remains after menstruation and undergoes only limited changes during the menstrual cycle (Aplin & Singh, 2008). Cyclical ovarian function is essential for the regeneration of endometrium ensuring endometrial receptivity in the next cycle. On an average, a woman undergoes around 400 cycles leading to menstruation and about 2 pregnancies in her reproductive life (Teklenburg *et al.*, 2010). Failure to acquire a receptive phenotype can lead to subfertility and an impaired decidual response can lead to pregnancy complications such as pregnancy loss.



1. Single-layered epithelium
2. Basal lamina
3. Uterine glands
4. Connective tissue
5. Blood vessels



- A. Superficial functional layer
- B. Basal layer
- C. Myometrium

**Figure 1.1:** An Illustration of Human Endometrium. The inset shows cells of the single layered prismatic epithelium, the basal lamina and the blood vessels are shown enlarged. Also seen are few glands which reach the myometrium. Figure adapted from Human Embryology Online course in embryology for medicine students developed by the universities of Fribourg, Lausanne and Bern (Switzerland) with the support of the Swiss Virtual Campus

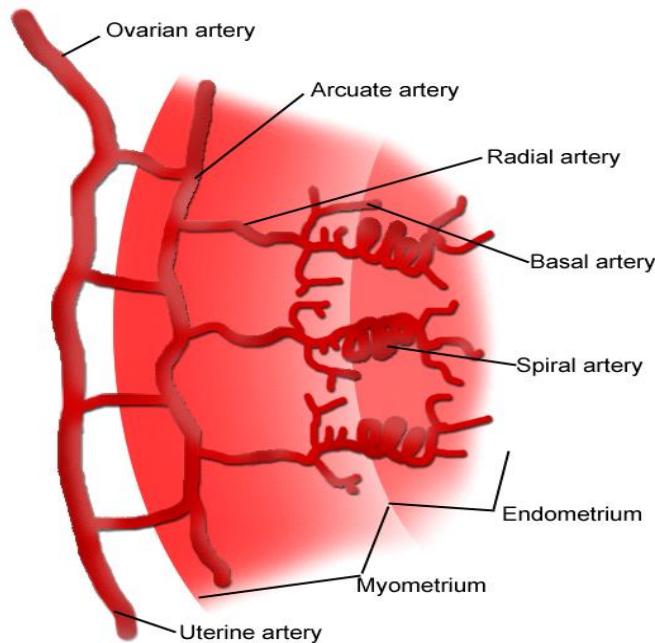
### 1.1.1 Structure of the endometrium

The uterus is a pear-shaped, muscular, hollow organ with a triple-layered wall with an outer serosal surface, the fibromuscular myometrium and an inner complex mucosal endometrium (Cooper, 2000). The endometrium consists of a single-layered simple columnar epithelium with or without cilia (depending on how far along the menstruation cycle is) and its basal lamina, uterine glands, and a specialised cell-rich connective tissue (stroma)

containing a rich supply of blood vessels (Gilbert, 2000). Invaginating from the mucosal surface deep into the stroma are the tubular uterine glands lined with columnar epithelium. The uterus derives its blood supply from the uterine arteries, which pass through the myometrium and splits into two types of endometrial vessels (Rogers, 1996). The arteries that cross the uterine walls and are responsible both for the myometrial and the endometrial blood supply are named radial arteries. As their minor branches arrive close to the myometrial-endometrial junction, they give rise to the *spiral* and *straight arteries*, which penetrate into the endometrium through its basal layer. The *straight arteries* are very short and small and they provide the blood supply exclusively to the endometrial basal layer. Their division into smaller arterioles, capillary and venous networks; follows the same pattern of almost all other tissues in the body.

On the other hand, the *spiral arteries*, which are highly sensitive to oestrogen and progesterone, penetrate the endometrial functional layer, grow and send branches within it and exhibit very different and unique patterns. They grow in length much faster than the whole endometrium grows in thickness. These arteries have to "accommodate" this growth by continuously accentuating their coiling. The spiral arteries give rise to two types of smaller arterial branches: 1) the common ones (similar to all others in the body), which gives rise to the arteriovenous capillary plexuses and networks that nourish the functional endometrial layer; 2) arteriovenous anastomosis, through which branches of the spiral arteries merge directly into the endometrial veins, constituting a kind of vascular "shunt", without any previous division into

capillaries. As to the endometrial veins, they exhibit dilations named "venous lakes."



**Figure 1.2:** The Uterine blood supply (source: <http://emedicine.medscape.com/article/1949215-overview>)

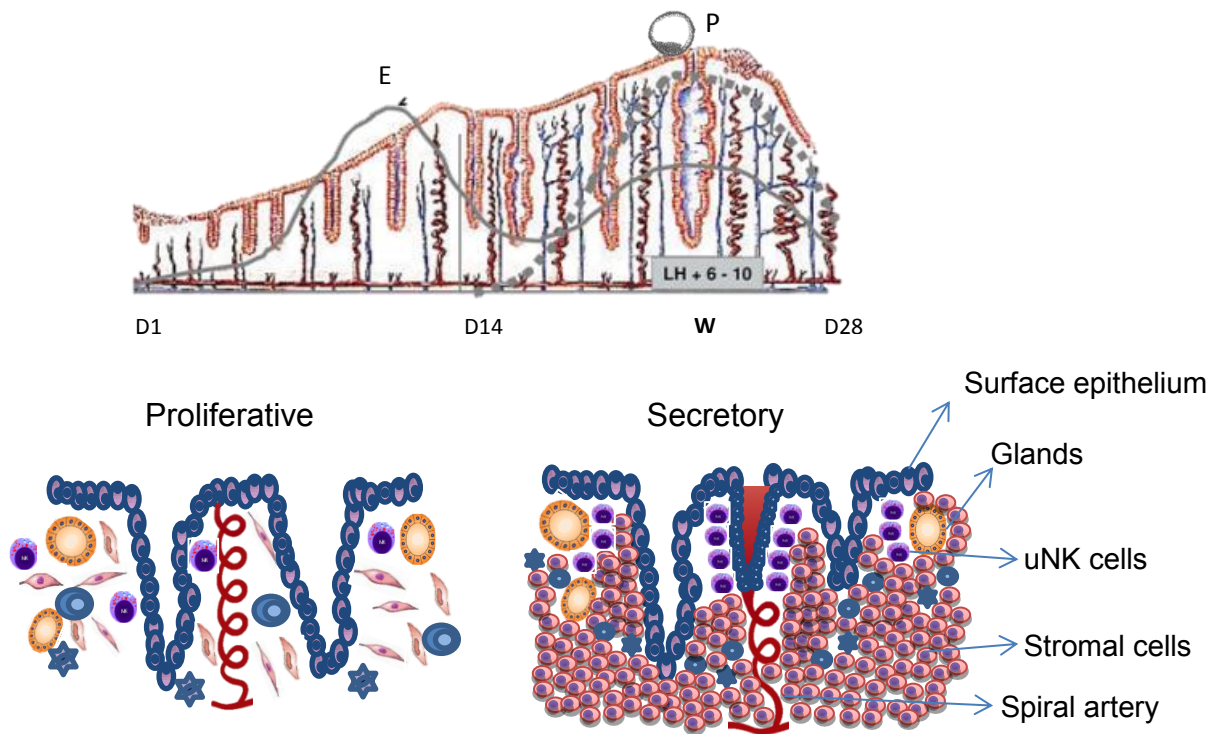
### 1.1.2 Cyclical changes of the human endometrium

The human endometrium is a unique dynamic tissue which undergoes cycles of tissue remodelling which includes proliferation, differentiation, recruitment of inflammatory cells, apoptosis, tissue breakdown and repair under the influence of sex steroid hormones, oestrogen and progesterone (Chiazze *et al.*, 1968; Al-Sabbagh *et al.*, 2012; Aitken *et al.*, 2008). After menstruation (days 1-5), the endometrium is a thin layer made up of the stratum basalis, containing the lower portions of the glands and spiral arteries. The circulating ovarian steroid hormones, both oestrogen and progesterone are low during menstruation which abolishes the negative feedback; hence the anterior

pituitary resumes gonadotrophin secretion. Follicular recruitment in the ovary occurs under the influence of these peptide hormones (Mihm *et al.*, 2011) secreted by the anterior pituitary (Mihm *et al.*, 2011). The developing follicles secrete oestrogen from granulosa cells, which in turn stimulates the remnant basal layer of the endometrium resulting in the proliferative phase. This phase lasts between 5-13 days. Epithelial cells rapidly proliferate to reconstitute and migrate to the surface to cover the denuded endometrial surface (Mihm *et al.*, 2011). During this phase, the stromal cells undergo proliferation as seen by the mitotic divisions and the vascular endothelial cells proliferate causing the spiral arteries to lengthen and grow into the replenishing endometrium (Gellersen *et al.*, 2007) and remain relatively straight. At the start of the proliferative phase, the glands extend only slightly into the stratum functionalis from the basal layer, however, by the late proliferative phase they would have extended onto the endometrial surface. As this phase progresses, these tubular glands become larger and more convoluted and mitotic figures appear. The endometrium grows from 1-2mm thick in the early proliferative phase to 5-6mm by the time of ovulation (Rogers, 1996). The surface and glandular cells acquire cilia and microvilli, which increase the cell surface area in order to improve the secretory and absorptive functions of the tissue.

The increasing circulating oestrogen levels trigger the luteinising (LH) surge by day 14 which stimulates ovulation (Mihm *et al.*, 2011). Ovulation marks the start of the progesterone driven secretory phase. Progesterone is secreted by the resultant corpus luteum following ovulation. The pre-receptive endometrium in the days following ovulation (days 15-19),

becomes receptive during the mid-secretory phase (days 20-23). The post-ovulatory rise in progesterone stops endometrial proliferation and induces differentiation or decidualization lasting for about 4 days (days 24-28) (Gellersen *et al.*, 2007; Gellersen & Brosens, 2003; Brosens & Gellersen, 2006). There is a dramatic remodelling of the endometrium along with characteristic cytoarchitectural changes in this phase. The glands become tortuous and sacculated with accumulation of glycogen, spiral arteries become increasingly coiled and there is influx of uterine natural killer (uNK) cells (Gellersen *et al.*, 2007). The stromal cells undergo transformation to decidual cells, with associated oedema along with changes in the extracellular matrix (ECM). The endometrium is now 7-18mm thick. The decidualizing endometrial stromal cells form a cuff around the changing spiral arteries (Craven *et al.*, 1998). The end of secretory phase is characterised by a thick stroma, which is highly vascularised with endometrial arterioles forming vascular clusters in the functional layer and absence of mitotic figures. This environment is favourable for blastocyst implantation. However, in the absence of a conceptus, the corpus luteum degenerates and the associated fall in progesterone levels induces spasmodic contraction of the spiral arterioles leading to ischemia of the functional layer. This layer is subsequently shed by menstruation (days 1-5).



**Figure 1.3:** Endometrial cycle under the influence of oestrogen and progesterone in proliferative and secretory phase respectively. Three different cell populations undergo changes from proliferative to secretory endometrium. The stromal cell population change from spindle shaped to more rounded decidual cells. The stromal cells become more confluent. There is increase in the number of uterine Natural Killer (uNK) cells in relation to the other leucocytes. The glandular cells undergo proliferation.

## 1.2 Menstruation

Menstruation is unique to a few species - the humans, old world primates, and wild fruit bats and elephant shrews (Zhang *et al.*, 2007). It is also interesting that decidualization of the endometrial stromal tissue in the absence of pregnancy occurs only in the menstruating species, thus implying a functional link between these two phenomena. Menstruation is defined as



the shedding of the superficial functional layer of the endometrium in response to the reduction in circulating progesterone leading to overt bleeding (Tabibzadeh, 1996). Earlier studies indicated that hypoxia and tissue destruction causes menstrual bleeding (Zhang & Salamonsen, 2002; Salamonsen, 2003). Recent studies have shown evidence that breakdown of endometrium is characterised by apoptosis, loss of decidual features, infiltration of inflammatory cells (neutrophils and mast cells) (Lathbury & Salamonsen, 2000) and activation of metalloproteinase (MMP) (Salamonsen & Woolley, 1999). Also, local production of prostaglandins causes vasospasm of the spiral arterioles and subsequent ischemia and sloughing of the endometrium (Henriet *et al.*, 2012).

### **1.2.1 Purpose of menstruation**

Menstruation is thought to have a more defined purpose rather than just an evolutionary accident. It is thought that cyclic endometrial decidualization and menstrual shedding is an example of physiological preconditioning that prepares uterine tissue for the dramatic vascular remodelling, reactive oxygen species production and hyperinflammation associated with deep hemochorial placentation (Brosens *et al.*, 2009). Menstruation and pregnancy are both inflammatory events albeit of very different magnitudes. Both events are associated with variable degrees of uterine free radical production, oxidative stress, ischemia-reperfusion injury, vascular remodelling, and angiogenesis (Jennings *et al.*, 1990; Otani, 2008). Therefore, it seems not only plausible but likely that cyclic endometrial

remodelling and menstruation preconditions the human uterus for pregnancy (Brosens *et al.*, 2009).

### **1.3 Decidualization**

In humans and in a small handful of species, decidualization takes place independent of conception. This is a progressive process occurring in the late mid secretory phase in a temporal and spatial fashion comprising of morphologic, biochemical, and vascular modifications initiated by the presence of progesterone after oestrogen priming (Ramathal *et al.*, 2010).

The implanting blastocyst initially apposes and attaches to the luminal epithelium of the endometrial lining about 6–7 days after conception. Soon after the epithelium is breached, the interface lies between trophoblast and decidual cells. The trophoblast invasion extends beyond the endometrium into the uterine junctional zone, which is the inner third of the myometrium (Brosens *et al.*, 2010; Brosens *et al.*, 1995). The decidua–trophoblast dialogue orchestrates the remarkably dynamic process by which the blastocyst becomes completely embedded in the uterine wall within a few days, as early as 10 days after LH surge (Norwitz *et al.*, 2001; de Ziegler *et al.*, 1998). A defect predisposes to related pregnancy complications, including miscarriage, preeclampsia, fetal growth restriction, and preterm labour. Inadequate uterine receptivity is thought to be responsible for two-thirds of implantation failures (Simón *et al.*, 1998).

Decidualization is characterized by transient local oedema, influx of macrophages and specialized uterine natural killer cells, angiogenesis and the extraordinary transformation of resident endometrial stromal fibroblasts

into secretory, epitheloid-like decidual cells (Gellersen *et al.*, 2007; Brosens *et al.*, 2009; Cloke *et al.*, 2008), first apparent in the vicinity of terminal spiral arteries and then spreads throughout the endometrial compartment.

Morphologically, the undifferentiated, elongated fibroblast-like endometrial stromal cells transform into enlarged round cells with specific ultrastructural modifications comprising of enlargement and rounding of nucleus, expansion of Golgi complex and rough endoplasmic reticulum along with cytoplasmic accumulation of lipid and glycogen droplets. The size increases by five times with an open vesicular nucleus and pale cytoplasm easily identified morphologically on standard haematoxylin and eosin (H&E) staining. Decidual cells develop multiple projections on the cell surface which extends into the ECM or indent adjacent cells and expresses desmin,  $\alpha$ -smooth muscle actin and vimentin signifying a myo-fibroblastic phenotype (Oliver *et al.*, 1999). There is emerging evidence that HESCs show an interesting mesenchymal-epithelial transition during decidualization (Li *et al.*, 2010); where mesenchymal cells show invasive and mobile properties and epithelial cells show cell-to-cell cohesion and integrity of multicellular organs forming barriers separating internal and external environments (Micalizzi *et al.*, 2010; Shook & Keller, 2003). Upon decidualization, HESCs expresses a myriad of genes characteristic of epithelial cells (Gellersen *et al.*, 2007).

Two of these genes, IGF-binding protein-1 (*IGFBP-1*) and prolactin (*PRL*), are preferentially induced in HESCs during decidualization and are therefore recognized as specific markers of decidualization (Gellersen & Brosens, 2003; Giudice *et al.*, 1993). There is concomitant laying of a peri-cellular rim

of extracellular matrices such as laminin, type IV collagen, fibronectin and heparin sulfate proteoglycan as part of their differentiation program (Dunn *et al.*, 2003; Tabanelli *et al.*, 1992). It is unusual for basement like extracellular proteins to surround mesenchymal cells in this manner (Loke *et al.*, 1995). This rim of matrix along with IGFBP-1 is thought to provide the scaffold for trophoblast cells to move over in their passage towards the arteries and myometrium.

### **1.3.1 Functions of the decidua**

Under the influence of progesterone, endometrial stromal cells become specialized epithelioid cells thus acquiring a secretory phenotype, which regulates numerous biological functions including trophoblast invasion, immunomodulation, hemostasis, angiogenesis and oxidative stress defence mechanisms, all of which are essential in ensuring a successful pregnancy. However, in the absence of pregnancy, the progesterone levels decline which in turn activates a sequence of events leading to tissue breakdown of the superficial endometrial layer and menstrual bleeding (Brosens & Gellersen, 2006). Both implantation and menstruation are considered as inflammatory events characterized by leukocyte infiltration and increased inflammatory mediator expression in endometrium.

### 1.3.2 Progesterone and cAMP signalling Pathways

#### Progesterone

Progesterone, estrogen and cyclic adenosine monophosphate (cAMP) together regulate the decidualization of human endometrial stromal cells in a time-dependent manner. Decidualization *in vitro* can be achieved by separating primary HESC's and by culturing them with a combination of synthetic progesterone (medroxy progesterone acetate; MPA) and cAMP analogue (8-Bromo-cAMP; 8-Br-cAMP). The ovarian hormones progesterone and estrogen are essential for decidualization and mainly act through their respective receptors, i.e. progesterone receptors (PRs) PR-A and PR-B and estrogen receptors (ERs) ERa and ERb. The androgen receptor (AR) is also involved in decidualization (Cloke *et al.*, 2008). Knock-out studies in mice have shown the absence of decidualization and implantation when they lack PR-A and PR-B (Lydon *et al.*, 1995). PR-A is required to inhibit the oestrogen induced hyperplasia and limit the proliferative effects of PR-B (Mulac-Jericevic *et al.*, 2000). Upon decidualization, HESC's show down regulation of PR-B, rendering the PR-A as the dominant isoform (Mote *et al.*, 1999; Wang *et al.*, 1998).

Progesterone treatment alone induces a slow and minimal differentiation response in HESC's taking up to 10days. Treatment with 8-Br-cAMP, the cell permeable analogue gives an immediate effect, although not sustained in long term cultures (Brosens *et al.*, 1999).

## Cyclic AMP signalling

The *in vivo* activity of adenylate cyclase and cyclic adenosine monophosphate (cAMP) levels are higher in the endometrium than the myometrium and during the secretory phase (Tanaka *et al.*, 1993). The cAMP signalling cascade is activated by the progesterone dependant decidualization of the stromal cells (Brar *et al.*, 1997). Cyclic AMP was thought to be the initial activator of decidualization and to be crucial for the slow-acting progesterone pathway (Tang & Gurpide, 1993). There seems to be more than one mechanism by which cAMP signalling sensitises HESC's to progesterone. Cyclic AMP initiates decidualization with a sustained activation of the protein kinase A (PKA) pathway that sensitizes the stromal cells to the activity of progesterone (Gellersen & Brosens, 2003). The Luteinising hormone (LH) surge and the follicle stimulating hormone (FSH) peak stimulate cAMP at first (Tang & Gurpide, 1993) and then relaxin, corticotropin-releasing hormone, prostaglandin E2 or human chorionic gonadotropin from trophoblasts take over the stimulation of cAMP until term if pregnancy occurs (Houserman *et al.*, 1989; Tang *et al.*, 1994; Telgmann & Gellersen, 1998; Makrigiannakis *et al.*, 1999).

## 1.4 Endometrial receptivity and window of implantation

In placental mammals, the uterus differentiates into an altered state when implantation-competent blastocysts are ready to initiate implantation. Successful implantation is a subtle dialogue between the maternal endometrium and the embryo. A receptive endometrium is a prerequisite

from the maternal side (Giudice, 1999a) which lasts for a limited period of time. The luminal epithelium undergoes precisely defined morphological changes until a receptive endometrium is developed. These changes were described as early as 1950 by Noyes, Hertig and Rock, and occur under the control of sex steroid hormones, oestrogen and progesterone. Uterine sensitivity to implantation is classified into prereceptive, receptive and nonreceptive (refractory) phases. During the prereceptive phase, the uterus is unable to initiate implantation; however the uterine environment is less hostile to blastocyst survival. In contrast, during the refractory phase, the uterine environment is unfavourable to blastocyst survival. The average length of the human menstrual cycle is 28-30 days. During the secretory phase, the uterus is considered prereceptive for the first ~7 days following ovulation (day 0). Following this, days 7-10, the uterus becomes receptive, also known as 'Window of Implantation'. The nonreceptive (refractory) phase comprises the rest of the secretory phase. The 'implantation window' is described as the period in the mid-luteal phase from day 19 to day 24, when implantation can take place (Navot *et al.*, 1991; Dominguez *et al.*, 2003) and is time limited (Wilcox *et al.*, 1999). This corresponds to the time when the embryo hatches, 6 days after LH surge, and consequently ready for implantation within the following 24 hours (Aplin & Singh, 2008). This synchronisation is also thought to serve as the mechanism to select against the developmentally compromised embryos (Teklenburg *et al.*, 2010b). Also, Wilcox *et al.* in 1999, demonstrated that implantation beyond the period of endometrial receptivity is strongly associated with early pregnancy loss (Wilcox *et al.*, 1999). Reduced endometrial receptivity has been found in an

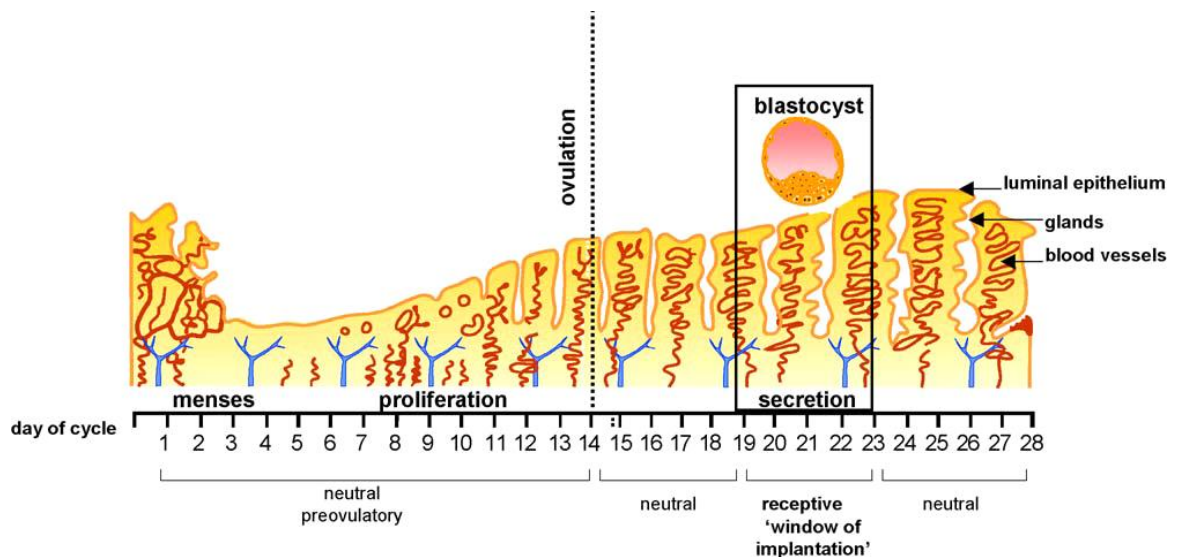
increasing number of unexplained infertilities (Lessey *et al.*, 1995). Adequate development of endometrium in the proliferative phase under the influence of rising oestrogen is crucial for synchronisation of the maturation process in the progesterone mediated secretory phase which is necessary for implantation (Hoozemans *et al.*, 2004). Opening of the implantation window is characterised by remarkable ultra-structural changes in the endometrial epithelial cell morphology which includes cytoplasmic protrusions of the endometrial surface arising from the apical surface of epithelial cells extending into the uterine cavity, known as pinopodes (Nikas, 1999). The pinopode formation and maintenance is dependent on progesterone (Stavreus-Evers *et al.*, 2001). The timed correlation of pinopode expression, period of blastocyst hatching, along with the preference of the blastocyst to attach to pinopodes suggests that pinopodes are structural markers of the receptive endometrium (Aghajanova *et al.*, 2008; Achache & Revel, 2006).

Like every phase of the menstrual cycle, this phase of receptivity is no doubt orchestrated by many known and unknown genes regulated by autocrine, paracrine and juxtacrine factors. A number of molecules have been identified to be involved in this process, such as integrins and their ligands (e.g. osteopontin), mucins, growth factors (HB-EGF), cytokines (LIF, leptin, IL-1, IL-11, IL-15), homeobox transcription factors (HOXA gene products), lipids and other molecules (Aghajanova *et al.*, 2008; Giudice, 1999b; Wang & Dey, 2006).

Since the development of microarray technology (Schena *et al.*, 1995), many researchers have attempted to profile the endometrium in different phases of



the cycle. Each study has provided a list of candidate genes (Carson *et al.*, 2002; Kao *et al.*, 2002; Borthwick *et al.*, 2003; Mirkin *et al.*, 2005; Riesewijk *et al.*, 2003), but the number of common genes for 'endometrial receptivity' is small (Horcajadas *et al.*, 2007). This poor consistency was possibly due to differences in the experimental design, data analysis, timing of the endometrial sample in the menstrual cycle, differences in the geographical location of the subjects and different statistical methods employed. Despite all these differences, molecular and genetic evidence indicate locally produced signalling molecules, including cytokines, growth factors, homeobox transcription factors, lipid mediators and morphogens, together with ovarian hormones specify the uterine receptivity and impaired expression of these receptivity genes is increasingly linked to common reproductive disorders. Although the luminal endometrial epithelium is the primary barrier in the implantation process, the progesterone responses in this cellular compartment that underpin the receptive phenotype are mediated by signals derived from the underlying stromal cells (Simon *et al.*, 2009) i.e. from decidualization of the stromal compartment.



**Figure 1.4:** Endometrium in menstrual cycle showing the ‘window of implantation’ or receptive period

## 1.5 Implantation

Implantation is a dynamic process involving embryo apposition, adhesion to the endometrial epithelium and encapsulation into the stroma (Loke *et al.*, 1995). This period spans from the time of blastocyst hatching to the formation of a primitive placental circulation system. The process is initiated upon contact between the blastocyst and endometrium.

### 1.5.1 Pre-implantation embryo development

Within 24 hours of fertilization, the zygote undergoes a regulated series of mitotic cell divisions. It is transported actively through the fallopian tube by the cilia and muscular contractions into the endometrial cavity, which lasts for four days. (Croxatto, 2002) At 4 cell stage, the genomic activation starts to switch from maternal to embryonic transcription (Duranthon *et al.*, 2008).

The embryo then follows the 8 cell stage, the blastomere stage, the morula stage (16cells) and then the blastocyst before implantation. The trophoblast cells (outer cell layer) undergo compaction, connected by tight junctions and microvilli with synchronous formation of a blastocyst cavity. The blastocyst will then hatch from the zona pellicuda, which is a glycoprotein membrane surrounding the oocyte. The blastomere cells are totipotent with the ability to differentiate into any cell type in the developing embryo. The first cells to attach and invade the endometrium are trophectoderm cells (Meseguer *et al.*, 2001). The trophoblasts produce a number of cytokines, growth factors like human chorionic gonadotrophin (hCG), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukins, which signal the maternal tract to promote implantation (Chen *et al.*, 2005; Merviel *et al.*, 2001). The receptive endometrium responds to these signals leading to the cross-talk between the trophoblast cells and the endometrium mediated in an autocrine and paracrine manner. This cross-talk requires many molecules including integrins, matrix-degrading enzymes, their inhibitors, growth factors, cytokines and their receptors, and modulator proteins (Giudice, 1999b; Nardo *et al.*, 2003b; Nardo *et al.*, 2003a).

About 60% of all pregnancies in the pre-implantation period are unsuccessful due to various factors like poor quality embryo, chromosomal abnormalities and mosaicism (Brosens & Gellersen, 2010). Mosaicism however decreases drastically in the cleavage stage embryo (Santos *et al.*, 2010).

### 1.5.2 Implantation process

Implantation occurs 6-7 days post fertilization (Croxatto, 2002). On apposition of the embryo to the endometrial surface, there is binding of the L-selectin expressed on the trophoblast with the oligosaccharide ligands on the luminal epithelial cells and a loose connection is established (Fazleabas & Kim, 2003). There is active communication between the trophoblast and endometrium conveyed by receptor-ligand-interactions (Aplin & Kimber, 2004). After adhesion, trophoblast cells differentiate into syncytiotrophoblast on the outside and cytotrophoblast on the inside and invasion starts with the lytic activity of the syncytiotrophoblast weakening the endometrial structures enabling penetration. The blastocyst then penetrates the endometrial luminal epithelium into the endometrial stroma up to the inner third of the myometrium. The endovascular extravillous trophoblast cells invade the terminal spiral arteries, plugging them for several weeks before destroying the musculoelastic wall thus establishing high-flow, low resistance uteroplacental circulation (Grewal *et al.*, 2010). By day 9, the embryo is completely embedded in the endometrium. Now the balance between the trophoblast invasion and the maternal restraint on invasion is essential to prevent detrimental effects on the mother (Emera *et al.*, 2012). By day 10, the cytotrophoblast invades the entire endometrium and the inner third of the myometrium. The endometrium also has a selective role by being able to dispose the developmentally compromised embryos by triggering a menstrual like event (Teklenburg *et al.*, 2010b). Increased vascular permeability and neoangiogenesis mediated by vasoactive substances like

vascular endothelial growth factor (VEGF) and angiopoietin are critical for early placentation.

For a successful implantation, there needs to be a co-ordinated development of a good quality embryo, a receptive and a selective endometrium specifically during the implantation window.

## **1.6 Immunomodulation**

The father of reproductive immunology, Peter Brian Medawar in 1953, for the first time described the “paradox of pregnancy”, (Medawar, 1953). Intriguingly, the fetus escapes maternal immune rejection even though it is a semi-allograft comprising of both paternal and maternal genetic components. This pivotal question led to the birth of many studies looking at the immune cells in reproduction.

### **1.6.1 Immune cells in the endometrium**

Leukocytes comprise approximately 30–40% of decidual stromal cells in early human pregnancy. During decidualization, there is an influx of innate immune cells. The population of immune cells in the endometrium and decidua are distinctly different to that of peripheral blood (Bulmer *et al.*, 1991; Johnson *et al.*, 1999). In particular, there are virtually no B cells and very few neutrophils (Salamonsen & Lathbury, 2000). Three main cell types are seen: T cells, macrophages and uNK cells (also called large granular lymphocytes) (Bulmer *et al.*, 1988) The numbers and proportions of each cell type vary through the menstrual cycle and in early pregnancy. Approximately 45% of

leukocytes are made of T cell leukocytes in the proliferative endometrium. Their absolute numbers remain constant throughout the cycle and in early pregnancy, however, their relative numbers decrease as the proportion of uNK cells increase (Bulmer *et al.*, 1991). Macrophages make up approximately 15-20% of endometrial leukocytes; their numbers increase slightly during the secretory phase of the cycle and early pregnancy so that they comprise 20% of leukocytes in the placental bed in early pregnancy (Bulmer *et al.*, 1988). The uNK cells however show cycle dependant changes.

### **1.6.2 Peripheral Natural Killer (NK) cells**

Mature NK cells constitute 5-20% of the total lymphocyte population in the peripheral blood. There are two subsets of NK cells, based on their expression of receptors CD56 and CD16 (Moffett-King, 2002). The subset in the peripheral blood expresses CD56<sup>dim</sup> CD16<sup>+</sup> and has cytotoxic and lytic activities. NK cells populate different peripheral lymphoid and non-lymphoid organs including lymph nodes, thymus, tonsils, spleen and uterus. (Freud & Caligiuri, 2006; Vosshenrich *et al.*, 2006).

### **1.6.3 Human uterine Natural Killer (uNK) cells**

The presence of granulated cells in the human endometrium was first noted in 1920. The cells were characterised by the presence of phloxinophilic cytoplasmic granules in the phloxine tartrazine stain (HAMPERL &

HELLWEG, 1958; Dallenbach *et al.*, 1987). The phenotype of the uNK cells differ from the usual peripheral blood NK cells by being CD56<sup>bright</sup> and CD16<sup>-</sup>. There is a small subgroup of peripheral blood NK cells with this phenotype. However, the CD56<sup>bright</sup> NK cells in the peripheral blood is different to the CD56<sup>bright</sup> uNK cells in that those in the peripheral blood are less granular and less cytotoxic than the CD56<sup>dim</sup> NK cells (Cooper *et al.*, 2001) whereas the uNK cells have characteristically abundant cytoplasmic granules and at least in some studies are able to efficiently lyse NK targets (Jones *et al.*, 1997). The uNK cells also differently express NK<sub>p</sub>30, NK<sub>p</sub>44 and NK<sub>p</sub>46, whilst the peripheral blood NK cells express NK<sub>p</sub>30 and NK<sub>p</sub>46 (Ritson & Bulmer, 1987; El Costa *et al.*, 2009). They differ from the majority of NK cells found in the peripheral blood; the majority express CD56 and CD38 but not the classical T-cell or NK-cell markers CD3, CD4, CD8, CD16 and CD57 (Bulmer *et al.*, 1991). The CD56 expression is tenfold higher than the peripheral cells. uNK cells are referred to as CD56<sup>bright</sup> and CD16<sup>-</sup> cells.

#### Terminology:

Many terms have been used for the granulated cells identified in the endometrium of many species, which are confusing and remain inconsistent. In humans, different names include: 'granular endometrial stromal cell' (HAMPERL & HELLWEG, 1958), 'endometrial granulocytes', based on their lymphocyte-like morphology, 'Körnchenzellen' or 'K' cells (Dallenbach *et al.*, 1987) and, more recently 'endometrial (or decidual) granulated (or granular) lymphocyte (or leucocyte)' (Bulmer *et al.*, 1991). With recognition of the diversity of NK populations in general and expression of NK receptors, it seems reasonable to adopt the term 'uterine NK cell', which emphasises that

these represent a special NK cell subgroup. In humans, uNK cells are not really 'uterine' but 'endometrial', being absent in myometrium.

#### Localisation and distribution of uNK cells

Uterine NK cells are a phenotypically distinct subset of NK cells and to date, similar cells have not been described in substantial numbers in other organs. Uterine NK cells have been identified in both non-pregnant endometrium and pregnant decidua. uNK cell numbers show the most dramatic menstrual cycle dependent changes. uNK cells are detected in the stratum functionalis in the late secretory phase and early pregnancy decidua, forming aggregates around spiral arteries and glands (Bulmer *et al.*, 1991). Their differentiation is triggered by decidualization. Their numbers are almost equal to the T cells in the proliferative phase with an increase in numbers during the mid-secretory phase of up to 70% of the endometrial leukocyte population and continue to increase further in early pregnancy (King *et al.*, 1989). Their numbers begin to decline by 14-16 weeks of pregnancy (Bulmer & Lash, 2005). There is little information about their distribution in the second trimester of pregnancy. However, there seems to be a decline in the uNK cell population to virtually nil at term (HAMPERL & HELLWEG, 1958; DALLENBACH-HELLWEG & NETTE, 1964).

#### Origin of uNK cells: Trafficking from blood or in situ differentiation of uterine NK cells?

It has been a matter of debate whether human CD56<sup>bright</sup> uNK cells arise from uterine progenitors or from other CD34<sup>+</sup> hematopoietic progenitor cells in the implantation site. There is evidence supporting both trafficking of differentiated cells from the peripheral circulation and in situ differentiation



and proliferation (Bulmer & Lash, 2005; Manaster & Mandelboim, 2010; Manaster & Mandelboim, 2008). Reports suggest strongly that at least a significant proportion, if not all, mature human uNK cells arise from extra-uterine progenitors in secondary lymphoid tissues and in the thymus (Manaster & Mandelboim, 2010).

In the late secretory endometrium and early pregnancy decidua, uNK cells are seen in the functional layer forming aggregates around the spiral arteries and glands (Bulmer *et al.*, 1991). This typical perivascular distribution was thought to be due to the diffusion of progesterone from the blood into this area. However, more recently, this is thought to reflect trafficking of uNK cells or their precursors from the circulation (Trundley & Moffett, 2004). The perivascular distribution could reflect a role in the remodelling of spiral arteries which is an essential feature of early pregnancy. There are however several arguments against the trophoblast induced trafficking of these cells. The uNK cells are seen prominently in the premenstrual endometrium in the absence of trophoblast and in the intrauterine decidua of ectopic pregnancy (von Rango *et al.*, 2001). Exogenous progesterone also shows a similar effect.

Human uNK cells are terminally differentiated cells found in the transient decidua. This striking association of the uNK cells with decidua is independent of the trophoblast. Even, areas of ectopic decidualization as seen on the ovarian surface or in the cervix in pregnancy invariably contain uNK cells. Comparably, these cells are seen in the intrauterine decidua of ectopic pregnancy (von Rango *et al.*, 2001; Vassiliadou & Bulmer, 1998). It

has been reported that peripheral blood NK cells from pregnant women had a greater migratory capacity through decidual endothelial cells and stromal cells than peripheral blood NK cells from males or non-pregnant women (Carlino *et al.*, 2008). Also, upon treatment of decidual stromal cells with progesterone, there is increased migration of NK. The peripheral blood NK cells acquire a chemokine receptor pattern similar to that of decidual uNK cells (Carlino *et al.*, 2008).

On the other hand, there is evidence that the uNK cells originate from *in situ* differentiation of immature progenitor cells from the circulation. There are no reports of substantial numbers of 'usual' NK cells in endometrium at any stage of the menstrual cycle or in early pregnancy. There is however a report of local differentiation of uNK cells in the mouse and CD34<sup>+</sup> positive (Bilinski *et al.*, 2008) as well as CD56<sup>+</sup> positive leukocyte stem cells in the secretory phase endometrium (Lynch *et al.*, 2007). Incubation of peripheral blood NK cells in TGFβ1 has been shown to lead to conversion into a uterine NK cell phenotype (Keskin *et al.*, 2007). Forty percent of CD56<sup>+</sup> uNK cells isolated from the late secretory phase endometrium express Ki67, a marker of mitotic activity (Jones *et al.*, 1998). Stromal mitoses are a recognised factor in premenstrual endometrium. This again suggests the possibility of local proliferation of uNK cells.

#### Uterine NK cells and decidual cells

The co-localization of uNK cells with the stromal cells is entirely independent of trophoblasts. Uterine NK cells have been detected in the intrauterine

decidua of ectopic pregnancies (Vassiliadou & Bulmer, 1998; King *et al.*, 1999) and in foci of endometriosis which have undergone decidual change (King *et al.*, 1999). The close association with decidualization suggests a possible role of progesterone in their regulation, however, uNK cells do not express progesterone receptors and are not functionally altered by progesterone (King *et al.*, 1996; Stewart, 1998). The cells have been shown to express oestrogen beta receptors, prolactin receptors and glucocorticoid receptors (Gubbay *et al.*, 2002; Henderson *et al.*, 2003). The influence of progesterone is therefore likely to be via the stromal cells. The perivascular distribution of uNK cells suggests a close association with HESC decidualization as this is the first area to undergo predecidual change in the late secretory phase (Dallenbach *et al.*, 1987). Also, the presence of uNK cells in the foci of endometriosis and in ectopic sites undergoing decidualization without prominent vascular supply leads to the possibility that the HESC's could have a role in the differentiation of uNK cell, similar to the role of bone marrow stromal cells in the NK cell differentiation (Rosmaraki *et al.*, 2001). Interleukin-15 (IL-15) is necessary for the generation of normal NK cell numbers in vivo and HESC's upon decidualization produce IL-15, at least in part apparently under progesterone control (Okada *et al.*, 2000; Kitaya *et al.*, 2000). It is therefore possible that uNK cell population is unique to the uterus and they differentiate from the precursor cells under the influence of HESC's under the influence of progesterone (Bulmer & Lash, 2005).

#### Function of uNK cells

The exact role of the transient uNK cell population has been of great interest to researchers. They were initially thought to be a potential threat to the fetus since early studies in mice showed increased numbers in abortion-prone animals (de Fougères & Baines, 1987). However, more recent studies suggest a positive role for uNK cells in the maintenance of pregnancy. Mice knockout studies have shown that the placentae of uNK cell deficient mice are hypertrophic and result in fetal death (Guimond *et al.*, 1997), thus showing that although they are equipped with cytotoxic killing machinery, they have limited killing activity. The suggested functional roles of uNK cells include: regulation of trophoblast invasion (uNK cells and macrophages), spiral artery remodelling (uNK cells) and immune tolerance (T cells, regulatory T cells and dendritic cells) (Bulmer *et al.*, 2010).

The uNK cells produce plenty of immunoregulatory cytokines like TNF  $\alpha$ , TGF  $\beta$ 1 and IFN  $\gamma$  (Interferon gamma) but the importance of these cytokines are not established (Bulmer & Lash, 2005). The tightly controlled invasion of uterine tissues and spiral arteries is an essential feature of pregnancy (Pijnenborg *et al.*, 1983). The uNK cells have been found to undergo apoptosis a few days prior to menstruation, but are maintained if pregnancy occurs and when rescued by hCG (King *et al.*, 1989; Koh *et al.*, 1995). This temporal and spatial distribution has led to speculation that they play a role in the control of trophoblast invasion. The role of cytokines and growth factors produced by uNK cells in the regulation of trophoblast invasion continues to be the interest of many studies.

In view of the spatial aggregation of uNK cells around the spiral arteries in early pregnancy and the fact the uNK cells are a major source of angiogenic factors (Lash *et al.*, 2006; Hanna *et al.*, 2006), recently, a new role has been proposed for these cells in the remodelling or transformation of the uterine spiral arteries in conjunction with the trophoblasts (Pijnenborg *et al.*, 2006; Kam *et al.*, 1999). Further evidence is provided from mouse studies that uNK cells are important for vascular remodelling in pregnancy although changes comparable with those in human pregnancy are not seen in mice (Croy *et al.*, 1997). This reflects a role for uNK cells in the mediation of uterine vascular changes rather than in control of trophoblast invasion.

Where do uNK cells go?

The uNK cells decrease by the end of the menstrual cycle and in late pregnancy. It has been reported that the uNK cells undergo death by apoptosis (Kusakabe *et al.*, 1999; Hammer & Dohr, 1999). However, there is no evidence of apoptosis on Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) studies (Jones *et al.*, 1998). The other possibility raised is that uNK cells degranulate in late pregnancy (Spornitz, 1992). There is no definite evidence regarding the fate of uNK cells.

uNK cell population in Recurrent Miscarriage (RM)

Recurrent miscarriage is defined as the loss of three or more consecutive pregnancies in the first trimester of pregnancy affecting approximately 1% of the population (Regan & Rai, 2000). The cause of repeated pregnancy loss is multifactorial. This could be embryological like abnormal embryonic

karyotype or maternal like causes which affect the endometrium and/or placental development (Aplin, 2000; Li *et al.*, 2002). Maternal factors thought to contribute to recurrent miscarriage include coagulation disorders, autoimmune defects, endocrine disorders and endometrial defects (Regan & Rai, 2000; Li *et al.*, 2002; Li, 1998). However, in half of the RM patients, no contributory factors are identified. It has been postulated that a proportion of these losses may be due to immune causes.

Several studies have shown increased numbers of CD56+, CD16+ NK cells in the peripheral blood of women with RM either prior to or during pregnancy compared with healthy fertile non-pregnant or pregnant controls (Aoki *et al.*, 1995; Kwak *et al.*, 1995). The peripheral CD56+ NK cell activity decreases in normal fertile women during the first trimester of pregnancy in contrast to the high activity of CD56+ NK cell activity in women with RM (Kwak *et al.*, 1995; Higuchi *et al.*, 1995). On the contrary, there was a decrease in the decidual CD56+ cells in placental tissues from spontaneous miscarriages in RM compared with tissue from spontaneous miscarriages in women without RM and women requesting termination (Yamamoto *et al.*, 1999; Quack *et al.*, 2001). However, three separate immunohistochemical studies have shown increased numbers of CD56+ cells in the non-pregnant endometrium of women with RM (Clifford *et al.*, 1999; Quenby *et al.*, 1999; Tuckerman *et al.*, 2007) and lower numbers were seen in women with RM who subsequently had a live birth compared to those who miscarried (Quenby *et al.*, 1999). An explanation for these differences could be that the increased number in the endometrium could be due to CD56+, CD16+ cells as suggested previously

by Lachapelle *et al.*, 1996 (Lachapelle *et al.*, 1996), while the decreased number reported in decidua could be the CD56<sup>+</sup>, CD16<sup>+</sup> population. This was supported by a study in 2002 which showed increased numbers of CD16<sup>+</sup> cells in early pregnancy decidua of women with RM (Emmer *et al.*, 2000).

#### **1.6.4 HLA compatibility**

A conception must be recognized as non-self in order to trigger an immunologic process that prevents the maternal immune system from rejecting it. Inadequate response of the maternal immune system to stimulation by paternal antigens, due to HLA sharing, has been implicated in recurrent pregnancy loss (Carp *et al.*, 1994) or recurrent implantation failure (RIF) (Elram *et al.*, 2005). Such inadequate response may involve the imbalance of T helper 1:T helper 2 (TH1:TH2) response, causing the maternal system to be more cytotoxic.

#### **1.6.5 CD3+ T cells**

CD3+ T cells are the second most abundant population of leukocytes within the endometrium and decidua. No differences in the numbers of CD3+ T cells in endometrium were found between RM and control women (Lachapelle *et al.*, 1996; Quenby *et al.*, 1999). There was no difference in their numbers between early pregnancy decidua from normal fertile women and women with RM (Yamamoto *et al.*, 1999; Quack *et al.*, 2001) and in decidua from normal pregnancies and after spontaneous abortion

(Vassiliadou & Bulmer, 1998). The studies have shown a shift towards a higher CD4+/CD8+ ratio in endometrial biopsies from women with RM (Quenby *et al.*, 1999; Lachapelle *et al.*, 1996).

### **1.6.6 Macrophages**

There was no significant difference in macrophage number in the first trimester decidua and in the decidua of spontaneous abortions between RM and control population (Quack *et al.*, 2001; Vassiliadou & Bulmer, 1996). However, in the non-pregnant endometrium of RM patients, there was an increase in macrophages compared to the endometrium of RM population who had a live birth (Quenby *et al.*, 1999).

### **1.6.7 Cytokines**

Many cytokines and chemokines have been suggested to have a role in human implantation {van Mourik, 2009, Embryonic implantation: cytokines`, adhesion molecules`, and immune cells in establishing an implantation environment}. Amongst those studied include Interleukin 6 (IL-6), Leukaemia inhibitory factor (LIF), Interleukin 11(IL-11) and Interleukin 15(IL-15).

#### **Interleukin 11(IL-11)**

IL-11 and LIF are members of the IL-6 family. IL11 is identified as a growth factor acting on multiple stages during hematopoiesis, synergizing with other factors (Du, 1994; Paul, 1990). Their biological roles include proliferation,



differentiation and cell survival, all of which are essential for blastocyst development and implantation (Du, 1994).

Both LIFR and IL11Ra are expressed by human endometrial epithelium and the ligands are upregulated during the receptive phase and secreted into the uterine lumen. Hence, they may act on the luminal epithelium to facilitate blastocyst attachment during implantation. They are found to increase adhesion of primary endometrial epithelial cells to fibronectin and collagen IV (Marwood, 2009) which are present on the blastocyst surface (Shimomura, 2006) as well as in the first trimester trophoblast (Kurosawa, 1985), respectively. Mice studies have shown that IL-11 is required for maturation of uNK cells.

#### Interleukin 15(IL-15)

Interleukin-15 (IL-15) is necessary for the generation of normal NK cell numbers *in vivo* and HESC's upon decidualization produce IL-15, at least in part apparently under progesterone control (Okada, 2000; Kitaya, 2000). It is expressed in the human endometrium, decidua and placenta (Kitaya, 2000; Verma, 2000). It is expressed in both the stromal and epithelial components of the endometrium. Before decidualization, it is expressed higher in the epithelial cells than stromal cells during most of the cycle and following decidualization the expression is higher in the stromal cells (Kitaya, 2000; Chegini, 2002).

### **1.6.8 Th1/Th2 balance in pregnancy**

Cytokines can be functionally divided into two groups: those that are pro-inflammatory and those that are essentially anti-inflammatory but that promote allergic responses. Th1-type cytokines tend to produce pro-inflammatory responses; interferon gamma being the main Th1 cytokine. In order to counteract excessive pro-inflammatory response, Th2-type cytokines are produced which includes interleukins 4,5,13 and interleukin10. In humans, Th1 cells predominate the non-pregnant endometrium especially in the proliferative phase (Gargett, 2007). It appears that pregnancy rejection is mediated by Th1 cytokines whereas Th2 response confers protection. Progesterone is found to drive the Th2 response, possibly acting as the switch from Th1 to Th2 activity at the maternal-fetal response (Piccinni, 1995).

## **1.7 Steroid hormone regulation of endometrium**

The human endometrium is a steroid responsive tissue undergoing cycles of sequential proliferation, differentiation, breakdown and repair. During this course, it prepares and allows implantation of a blastocyst and supports any resultant pregnancy. Implantation and menstruation are under the control of steroid hormones. During each menstrual cycle the endometrium is exposed to three distinct hormonal environments: the oestrogen dominated

proliferative phase; the progesterone dominated secretory phase; and the progesterone withdrawal immediately prior to and during menstruation.

### **1.7.1 Steroid receptor expression in the endometrium**

The endometrial tissue expresses receptors for oestrogen, progestin, androgen and glucocorticoids and this has been well documented by immunohistochemical studies. There are two isoforms of oestrogen receptors – ER $\alpha$  and ER $\beta$ . During the secretory phase, both ER $\alpha$  and ER $\beta$  are present in the glandular epithelium and endometrial stroma. The expression of ER $\alpha$  decreases in the glandular epithelium and stroma of endometrium in the secretory phase. ER $\beta$ 1 localisation is unaltered across the menstrual cycle, while ER $\beta$ cx/ $\beta$ 2 decreases in the glandular epithelium of the functional layer in the mid secretory phase (Critchley, 2001; Critchley, 2002; Taylor, 2000). ER $\beta$ 1 is expressed by the uNK cells (Henderson, 2003) and both ER $\beta$ 1 and ER $\beta$ cx/ $\beta$ 2 are present in the endometrial endothelium (Critchley, 2002).

Progesterone has two isoforms – PR-A and PR-B. PR-A is expressed in the secretory endometrial stroma and in first trimester decidua (Mote, 1999; Wang, 1998; Brosens, 1999). Both isoforms are reduced in the glandular epithelium in the secretory phase. The endometrial endothelium or uNK cells do not express PR; however both isoforms are expressed in the perivascular cells (Wang, 1998; Henderson, 2003; Perrot-Applanat, 1994).

Glucocorticoid receptor (GR) is expressed in the endometrial stroma, endothelium of endometrial blood vessels and uNK cells peaking at the time of menstruation (Henderson, 2003, McDonald, 2006, Bamberger, 2001).

Androgen receptor (AR) is expressed only in the endometrial stroma and detectable throughout the menstrual cycle and first trimester decidua with maximal expression in the proliferative phase (Burton, 2003, Horie, 1992, Milne, 2005).

### **1.7.2 Glucocorticoids**

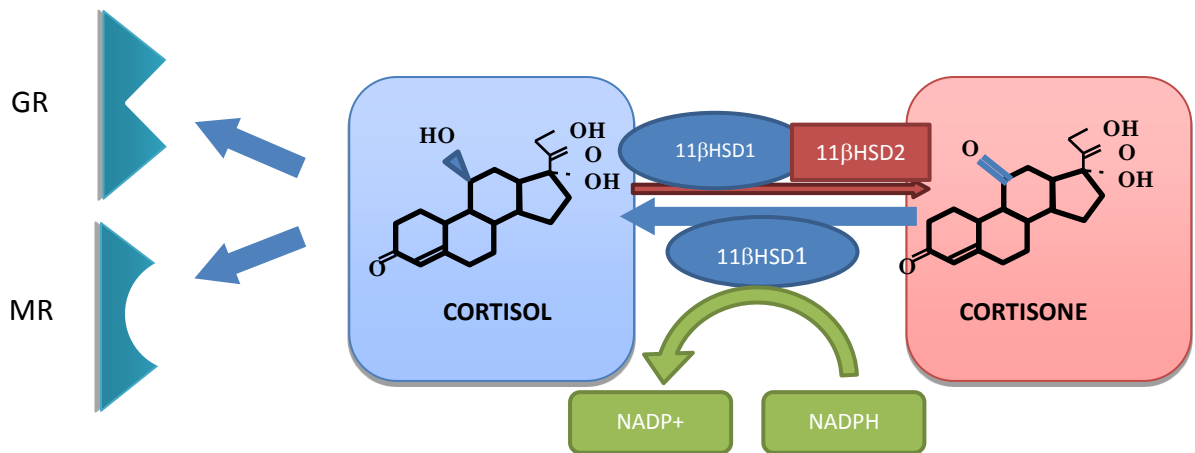
Glucocorticoids have been implicated in many processes that underpin successful embryo implantation, placentation, and the growth and development of the fetus (Michael, 2008).

#### **Metabolism**

Glucocorticoids play a vital role in the control of metabolism in all tissues and at a cellular level influences the amino acid, fatty acid and carbohydrate metabolism. Cortisol is the principal active glucocorticoid. Cortisol acts by binding to the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). A balance between synthesis and metabolism controls the circulating cortisol concentrations available to bind to these receptors. In the past, cortisol metabolism was thought to occur mainly in the liver, but we now know it occurs in many peripheral tissues including endometrium. The metabolism of cortisol plays a critical role in determining the activity of the hypothalamo-pituitary-adrenal (HPA) axis.

Medroxy progesterone acetate, a progestin used in many *in vitro* studies acts through the GR (Bamberger, 1999), and the antiprogestin RU486 acts as an antiglucocorticoid (Schatz, 1997).

The key enzyme in the metabolism of cortisol is 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD). By catalysing the interconversion of active glucocorticoid, cortisol and inactive cortisone, it is able to control the concentration of active glucocorticoid available to bind to GR. Furthermore, it is able to protect the MR from illicit occupation by cortisol (Tomlinson, 2001). Tissue levels of active endogenous glucocorticoids depend on the expression of 11 $\beta$ HSD enzymes. Although the 11 $\beta$ HSD1 isoform is a bidirectional enzyme, it predominantly catalyzes the conversion of inert cortisone (E) to active cortisol (F), thus increasing tissue levels of active glucocorticoids (Courtney, 2008). The reduced nicotinamide adenine dinucleotide [NAD(H)]-dependent type 2 isoform (11 $\beta$ HSD2) acts as a dehydrogenase, converting F to E (Ferrari, 1996). The main cellular targets for F are glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). However, GR normally co-localizes with 11 $\beta$ HSD1 *in vivo*, whereas 11 $\beta$ HSD2 is commonly found in MR-expressing tissues. Nevertheless, hypercortisolism can result in apparent mineralocorticoid excess as in Cushing syndrome. Further, some cell types like adipocytes do not significantly express 11 $\beta$ HSD2, thus enabling F to act through MR (Yang, 1997, Engeli, 2004).



**11βHSD:** 11β Hydroxy Steroid Dehydrogenase

**GR:** Glucocorticoid Receptor

**MR:** Mineralocorticoid Receptor

**Figure 1.5:** The 11βHSD isoenzymes: 11βHSD1 is a NADPH-dependent reductase which converts inactive cortisone to active cortisol and 11βHSD2 is a NAD-dependent dehydrogenase enzyme reverses the conversion from active cortisol to inactive cortisone

### Glucocorticoids and endometrium

Both the isoforms of 11βHSD as well as GR and MR are expressed in human endometrium (McDonald, 2006). Interestingly, GR expression is confined to stromal cells, whereas MR is reportedly present in both stromal and glandular compartments. Further, 11βHSD1, but not 11βHSD2, is highly up-regulated upon differentiation of primary human endometrial stromal cells (HESCs) into specialist decidual cells *in vitro* (Takano, 2007). This progesterone (P4)-driven differentiation process bestows unique functions on the endometrium that are essential for pregnancy, including the ability to

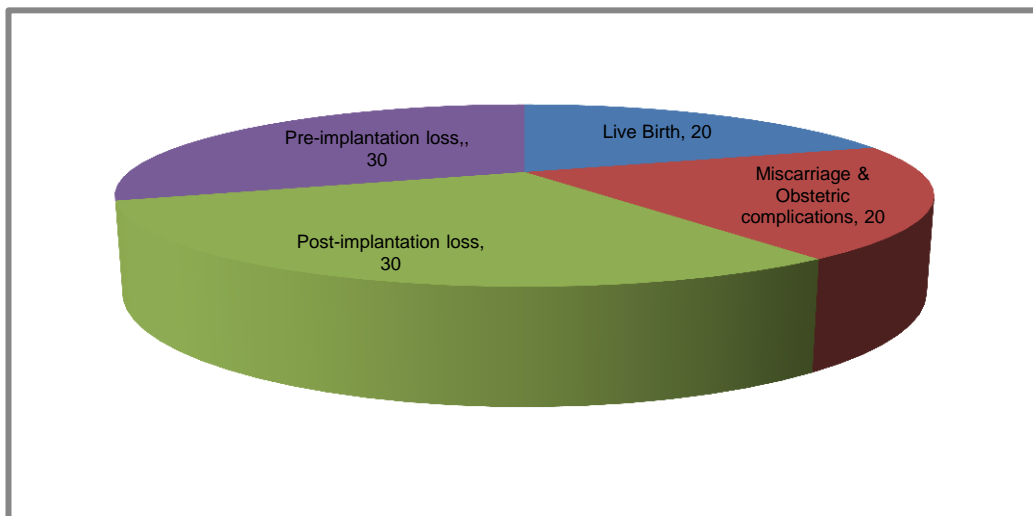
regulate trophoblast invasion, to modulate local angiogenesis, to recruit specialized uNK cells and macrophages, and to resist environmental and oxidative stress (Leitao, 2010, Gellersen, 2007). The speculation based on these observations, is that induction of 11 $\beta$ HSD1 would lead to increased F bioavailability, which in turn regulates the expression of distinct GR- and MR-dependent gene networks in decidualizing HESCs. Consequently, modulation of glucocorticoid action represents a potential strategy for the treatment or prevention of a variety of pregnancy-related disorders.

## **1.8 Clinical Perspective**

Human reproduction is remarkably inefficient compared to other mammalian species (Robillard, 2003, Preeclampsia and human reproduction. An essay of a long term reflection}. The probability of conception is 25-30% per cycle and only 50% of these conceptions continue beyond 20 weeks of gestation. Clinical miscarriage only accounts for 15% and, about 85% of total pregnancy losses are implantation failure and pre-clinical losses (Macklon, 2002) and are therefore not clinically recognized as pregnancies (Norwitz, 2001). Approximately 70% of spontaneous conceptions are lost prior to the completion of the first trimester of pregnancy. The pregnancy rate per cycle is even lower when in vitro fertilization (IVF) is employed in a natural cycle (Vlaisavljevic, 2007).

### 1.8.1 Reproductive failure

Reproductive failure is a broad term which includes the inability to conceive and the inability to maintain a pregnancy. This includes: Infertility, repeated implantation failure (RIF) in women undergoing In vitro fertilization (IVF) and recurrent miscarriage. Infertility is failure to conceive after regular unprotected intercourse after one year in the absence of reproductive pathology. Recurrent implantation failure is frequently defined as failure to achieve a pregnancy following 2-6 cycles of IVF with three fresh IVF attempts (Tan, 2005). Recurrent miscarriage is defined as the loss of three or more consecutive pregnancies prior to 20–22 weeks of pregnancy {Stirrat, 1990, Recurrent miscarriage}.



**Figure 1.6:** Overview of outcome of 100 conceptions



### **1.8.2 Infertility**

The prevalence of subfertility is high in humans affecting 10% of couples in the reproductive age. One in five to six couples in the UK suffer with this problem. Over 72 million women worldwide, aged 20-44, are currently sub fertile and every second couple seek medical care for subfertility (Boivin, 2007). Female fertility is regulated by a complex co-ordination and synchronization of interactions in the hypothalamic-pituitary-ovarian (HPO) axis; influenced by different diseases/ dysfunctions of the reproductive tract, neuroendocrine system, immune system or general disease (Evers, 2002). The known causes of female infertility include: ovulatory dysfunction, tubal disease, uterine factors and cervical factor. In 15-20% of cases, no cause is found and hence termed unexplained infertility. In 25-30% of couples, primary male factor infertility is diagnosed.

### **1.8.3 Recurrent Implantation Failure (RIF)**

Repeated implantation failure (RIF) is determined when embryos of good quality fail to implant following several in vitro fertilization (IVF) treatment cycles. Different fertility centres use different definitions; hence, the exact prevalence of RIF is difficult to determine (Laufer, 2012). RIF is frequently defined as failure to achieve a pregnancy following 2-6 cycles of IVF with three fresh IVF attempts (Tan, 2005) or more recently as failure to implant after at least three consecutive IVF attempts, in which 1–2 embryos of high grade quality are transferred in each cycle (Simon, 2012).

There are many variables that influence implantation of a potential embryo transferred in an IVF cycle including a hormonally primed “receptive” endometrium, hence any abnormality attributed to the embryo, the endometrium or the immune system will result in implantation failure. Increased numbers of CD56+ cells have been found in the endometrium of women with RIF after IVF (Lédée-Bataille, 2004; Lédée-Bataille, 2005).

#### **1.8.4 Recurrent miscarriage**

Three or more consecutive spontaneous miscarriages with or without previous live births are defined as recurrent miscarriage and this condition affects about 1–3% of women during their reproductive years (Carrington, 2005). The couple not only experience emotional and physical trauma, but live in anxiety of a further miscarriage when they conceive.

Primary RM refers to patients with consecutive losses and no prior successful pregnancy; secondary RM refers to losses following a live birth. Known risk factors for RM are genetic disorders, uterine pathologies, endocrine dysfunctions, autoimmune diseases, acquired and inherited thrombophilia as well as environmental factors (Rai, 2006). The cause for RM in 50% of patients is unknown (Li, 2002). The unexplained RM are clinically challenging and frustrating for the clinician. The prognosis is however good with a live birth rate of 75% following referral to a specialized clinic without any treatment (Clifford, 1997). Treatment of proven efficacy is available for only 15%. Recurrent or sporadic miscarriage is attributed to

either a chromosomal/ developmental abnormality in the embryo or due to uterine/endometrial factors.

### **1.8.5 Endometrium in RM**

The endometrium in RM appears to be 'super-receptive' allowing embryos of low viability to implant, presenting as a clinical pregnancy before miscarrying (Salker, 2010; Aplin, 1996; Quenby, 2002).

Higher numbers of uNK cells were found in the preimplantation endometrium of women with idiopathic RM compared to controls (Clifford, 1999; Quenby, 1999). Higher levels of uNK cells have been also been found in the decidua miscarried from women with RM compared with controls (Quack, 2001), and the decidua of RM patients were more active (Chao, 1995), and phenotypically different to those from healthy pregnancies (Emmer, 2002; Yamamoto, 1999). Higher CD56+ NK cells were found in the decidua from chromosomally abnormal miscarriages than in chromosomally normal miscarriages (Yamamoto, 1999). The decidual leucocytes from the miscarried tissue of women with unexplained RM and a normal fetal karyotype compared to women with RM and abnormal fetal karyotype (Quack, 2001).

Furthermore, high preconceptional peripheral NK activity was found to be predictive of further miscarriages in women with RM (Aoki, 1995; Ntrivalas, 2001) and women who had higher numbers of uNK cells were more likely to miscarry in a subsequent pregnancy (Quenby, 1999).

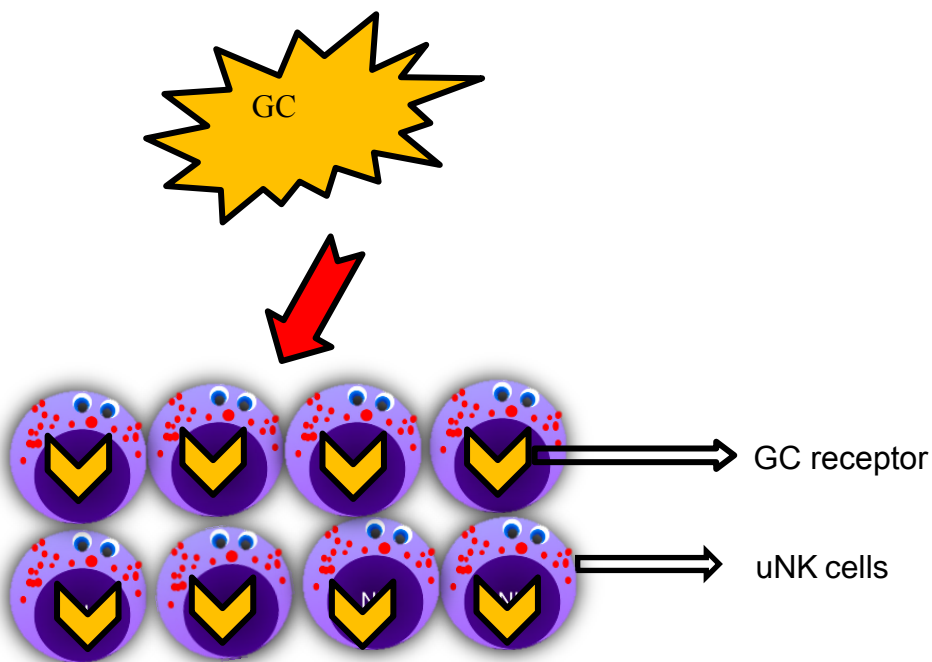
Henderson et al. (Henderson, 2003) found that uNK cells express the glucocorticoid receptor and the ER $\beta$ 1 receptor, thereby raising the possibility of immunomodulation of this cell population with pharmacological treatment. uNK cells express the glucocorticoid receptor (GR) but lack the progesterone receptor (PR), rendering them directly responsive to cortisol but not progesterone. Immunomodulation with IVIg, third-party donor cell immunization, paternal cell immunization, trophoblast membrane infusion and steroids were suggested. A Cochrane review in 2003 showed no beneficial effect from IVIg, third-party donor cell immunization, paternal cell immunization and trophoblast membrane infusions, after evaluating 19 trials (Scott, 2003). Quenby et al. (Quenby, 2005) showed that glucocorticoid (prednisolone) treatment can reduce the high numbers of uNK cells during the peri-implantation window.

### **1.8.6 Glucocorticoids in RM**

Glucocorticoids treatment has been advocated for the prevention of early pregnancy loss based on its ability to reduce the abundance of uNK cells during the peri-implantation window (Quenby, 2005), to stimulate human chorionic gonadotropin secretion by cultured human cytotrophoblasts (Ringler, 1989) and to accelerate trophoblast growth and invasion (Mandl, 2006).

However, implantation is an inflammatory process that depends on local release of pro-inflammatory cytokines and prostaglandins (Bazer, 2010; Chard, 1995; Kelly, 2001; Sharkey, 1998). By disabling the cytokine-

prostaglandin signalling cascade, glucocorticoids potentially could impact adversely on early pregnancy events. Furthermore, compelling evidence suggests that prolonged exposure to high levels of glucocorticoids in pregnancy is detrimental for both placental and fetal development (Aufdenblatten, 2009; Gennari-Moser, 2011; Seckl, 2004).



**Figure 1.7:** Schematic representation showing glucocorticoids acting on the uNK cells via the GC receptors

In summary, a receptive endometrium plays a pivotal role in implantation. A defect in the receptivity of endometrium is attributed to implantation failure and recurrent pregnancy loss. The expression of glucocorticoid receptors in the stroma of the endometrium and its role in decidualization is indeed intriguing. Interestingly, the finding of elevated number of uterine NK cells in the endometrium of recurrent implantation failure and recurrent miscarriage

and their expression of glucocorticoid receptor raises the possibility of immunomodulation of the endometrium in early reproductive failure.

## 1.9 Hypothesis and Aims

I postulate that the decidualization is closely associated with local corticosteroid metabolism regulated by the key enzyme 11 $\beta$ HSD and this is reflected by the uterine natural killer cell population in the sub-epithelial endometrium.

### Specific Aims:

- To study the expression and activity of 11 $\beta$ HSD1 in decidualizing human endometrial stromal cells and local cortisol biosynthesis.
- To study the glucocorticoid and mineralocorticoid pathways in the human endometrial stromal cells involved in local corticosteroid metabolism.
- To characterise the decidualization in patients with elevated uterine NK cell population in the sub-epithelial endometrial stromal cells by studying the decidual markers in timed endometrial biopsies.
- To establish that excessive uNK cell levels in mid-luteal endometrial samples reflects relative local corticosteroid deficiency possibly secondary to inadequate induction of decidual 11 $\beta$ HSD1 and thus resulting in impaired local metabolic function.
- To translate these findings to potential pharmacological immunomodulation of the impaired endometrium in women with early reproductive failure.

## **Chapter 2: Materials and Methods**



## 2.0 Materials and Methods

### 2.1 Materials

#### 2.1.1 Antibodies

##### Primary

<i>Antibody</i>	<i>Dilution</i>	<i>Manufacturer</i>
CD56(NCL-CD56-1B6) Lyophilized mouse monoclonal	1:200	Novocastra
Anti-HSD 11 B1(ab83522) Rabbit polyclonal	1:2000	Abcam
MCR (H-300): sc-11412 Rabbit polyclonal	1:1000	Santa Cruz
GR (E-20):sc-1003 Rabbit polyclonal	1:1000	Santa Cruz
RETSAT HPA007961 Rabbit polyclonal	1:1000	Sigma Life Science
DHRS3 15393-1-AP Rabbit polyclonal	1:1000	Protein Tech
RBP4 11774-1-AP Rabbit polyclonal	1:1000	Protein Tech

##### Secondary

Horseradish peroxidase(HRP) – conjugated goat anti-rabbit IgG	Dako
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##### Normal serum

Goat	Dako
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## 2.1.2 Cells

Endometrial cells	Tissue biopsy
HESC	Human endometrial stromal cells (primary)

## 2.1.3 Small interfering RNA (SiRNA)

ProFection mammalian transfection kit purchased from Promega was used.

The RefSeq gene accession numbers are as follows:

SiCONTROL nontargeting(NT) SiRNA pool

GR SiGENOME SMART pool SiRNA

MR SiGENOME SMART SiRNA

## 2.1.4 Culture media and materials

Dulbecco's modified eagles medium (DMEM)/F-12(1:1) 1x nutrient mixture, with L-Glutamine, 15mM HEPES± Phenol red	Invitrogen
Charcoal	Sigma
Collagenase type IA	Sigma
Dextran	Sigma
Deoxyribonuclease I (DNase I)	Roche
Foetal bovine serum (FBS) heat inactivated	Invitrogen
L-Glutamine, 200mM (x100)	Invitrogen
Penicillin (10,000U/ml)-streptomycin (10,000 micrograms/ml) solution (x100)	Invitrogen
Trypsin-EDTA solution (x1)	Invitrogen
Tissue culture plastic ware	Orange scientific Falcon Nunc Corning Starlab VWR

## 2.1.5 Reagents

Absolute Ethanol	VWR
Acetone	BDH
30% acrylamide/Bis solution (37.5:1)	Bio-Rad
Agarose powder	Appleton woods
Ammonium Persulphate (APS)	Sigma
Bovine Serum albumin (BSA) fraction V	Sigma
8-Bromoadenosine-3,5' – cyclic monophosphate sodium salt (8-Br-cAMP)	Sigma
Bromophenol blue	Sigma

## 2.1.6 Buffers and solutions

All buffers and solutions were made with deionised and distilled water. Solutions were stored at room temperature (RT), unless otherwise stated.

### Phosphate buffered Saline

140mM NaCl

2.5mM KCL

1.5mM  $\text{KH}_2\text{PO}_4$

10mM  $\text{Na}_2\text{HPO}_4$

### Tris Buffered Saline (TBS)

130mM NaCl

20mM Tris, pH 7.6

### TBS-Tween 20 (TBS-T)

0.1% Tween 20 in TBS

### Tris-Borate EDTA (TBE) Buffer (10x)

0.9 M Tris-Borate

2mM EDTA, pH 8.0

### Hanks Balanced Salt Solution (HBSS)

120mM mM NaCl

5.4mM KCL

0.8mM MgSO<sub>4</sub>

10mM HEPES

10mM Glucose

Adjust pH to 7.4 with NaOH

### HBSS with CaCl<sub>2</sub>

1.8mM CaCl<sub>2</sub> in HBSS buffer

Adjust pH to 7.4 with NaOH

### Dextran Coated Charcoal (DCC) suspension

1% (w/v) activated charcoal

0.1% (w/v) Dextran

### DNA Loading Buffer (10x) (Store at -20<sup>0</sup>C)

0.2% (w/v) Bromophenol blue

40% (v/v) Glycerol

0.25 M EDTA, pH 8.0

### Basic Lysis Buffer (Store at -20<sup>0</sup>C)

10mM Tris-HCl, pH 7.5

5mM EDTA

150mM NaCl

50mM NaF

30mM Sodium Pyrophosphate

10% (v/v) Glycerol

0.5% (v/v) TritonX-100

1 Complete Protease Inhibitor cocktail Tablet per 50ml of buffer

### Radio Immuno Precipitation Assay (RIPA) buffer (Store at -20<sup>0</sup>C)

1% NP-40 (v/v)

1% Triton X-100

150mM NaCl  
2mM NaF  
1% Sodium Deoxycholate (w/v)  
0.1% SDS (w/v)  
10mM Tris, pH 8.8  
1 Complete Protease Inhibitor cocktail Tablet per 50ml of buffer

#### High Salt Buffer

0.4m KCl  
20mM HEPES (pH 7.4)  
20% glycerol  
0.5mM PMSF  
1 Complete Protease Inhibitor cocktail Tablet per 50ml of buffer

#### Protein loading buffer (Laemmli Buffer) (Store at -20°C)

50mM Tris-HCl, pH 6.8  
50mM Imidazole, pH 6.8  
1% (w/v) SDS  
10% Glycerol  
2% (v/v) beta- mercaptoethanol  
0.002% (w/v) Bromophenol blue

#### Acid Alcohol Solution

1% HCl (v/v) in Ethanol  
*4% PFA Solution*  
4% PFA (w/v) in PBS  
Adjust to pH 7.4 with NaOH

#### Western SDS Running Buffer (10x)

250mM Tris Base  
1.9M Glycine  
1% (w/v) SDS  
*Western Transfer Buffer*  
250mM Tris Base  
192 mM Glycine, pH 8.3

20% (v/v) ethanol

### Western Blocking Buffer and Secondary Antibody Incubation Solution

5% (w/v) non-fat milk in TBS-T

*Western Primary Antibody Incubation Solution*

3% (w/v) BSA in TBS-T

### Western stripping Buffer

100mM beta-mercaptoethanol

2% (w/v) SDS

62.5mM Tris-HCl, pH 6.7

### SDS Polyacrylamide Gels

#### **Resolving Gels**

375mM Tris-HCl, pH 8.9

1% (w/v) SDS

10% (v/v) Acrylamide Bis Solution (37.5:1)

0.03% (w/v) APS

TEMED added at 1:1500

#### ***Stacking Gel***

80mM Tris-HCl, pH 6.8

80mM Imidazole

0.06% (w/v) SDS

5% (v/v) Acrylamide Bis solution (37.5:1)

0.06% (w/v) APS

TEMED added at 1:1500

## **2.1.7 Immunohistochemistry**

Immunofluorescence blocking solution

3% BSA (w/v)/ 10% normal serum (v/v) in PBS

Immunofluorescence Antibody dilution solution

3% BSA (w/v)/ 0.5-10% normal serum (v/v) in PBS

Immunohistochemistry Primary Antibody dilution solution

0.3% BSA (w/v)/ 10% normal serum (v/v) in PBS

Immunohistochemistry Secondary Antibody dilution solution

0.3% BSA (w/v)/ 1% normal serum

## **2.1.8 Enzyme Linked Immunoassay (ELISA)**

Quantikine kits used:

Human IL-15 Immunoassay (Catalogue Number D1500) consisting of the following:

- IL-15 Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against IL-15
- IL-15 Conjugate - 21 mL/vial of a mouse monoclonal antibody against IL-15 conjugated to horseradish peroxidase with preservatives
- IL-15 Standard - 2.5 ng/vial of recombinant human IL-15 in a buffered protein base with preservatives; lyophilized
- Assay Diluent RD1-19 - 11 mL/vial of a buffered protein base with preservatives
- Calibrator Diluent RD5-5 - 21 mL/vial of a buffered protein base with preservatives. For cell culture supernate samples
- Calibrator Diluent RD6-10 - 21 mL/vial of a buffered protein base with preservatives. For serum/plasma samples
- Wash Buffer Concentrate - 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives
- Color Reagent A - 12.5 mL/vial of stabilized hydrogen peroxide

- Color Reagent B - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine)
- Stop Solution - 6 mL/vial of 2 N sulphuric acid
- Plate Covers - Adhesive strips

Human IL-11 Immunoassay (Catalogue Number D1100) consisting of the following:

- IL-11 Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against IL-11
- IL-11 Conjugate - polyclonal antibody against IL-11 conjugated to horseradish peroxidase with preservatives
- IL-11 Standard - recombinant human IL-11 in a buffered protein base with preservatives; lyophilized
- Assay Diluent RD1S - buffered protein base with preservatives
- Calibrator Diluent RD5K - buffered protein base with preservatives

For cell culture supernate samples:

- Calibrator Diluent RD6N - animal serum with preservatives

For serum/plasma samples:

- Wash Buffer Concentrate - 25-fold concentrated solution of buffered surfactant with preservatives
- Color Reagent A - stabilized hydrogen peroxide
- Color Reagent B - stabilized chromogen (tetramethylbenzidine)
- Stop Solution - 2 N sulphuric acid
- Plate Covers - adhesive strips



## **2.2 Methods**

### **2.2.1 Ethics Approval and Patient Recruitment**

The study was approved by the University Hospitals of Coventry and Warwickshire Research ethics Committee (1997/5065). All women who participated in this study were fully informed through patient information leaflet and by the researcher. Written consent was obtained from all patients before endometrial sampling (Appendix 1).

### **2.2.2 Timed Endometrial samples**

Timed samples were collected from control group and the study group. The study group comprised of patients with idiopathic recurrent pregnancy loss (RPL). Idiopathic recurrent pregnancy loss (RPL) patients; where RPL is defined as three or more consecutive pregnancy losses before 24 weeks gestation and the cause is not known. The control group comprised of women with unexplained infertility; where unexplained infertility is defined as delay in conception after 12 months of unprotected intercourse after excluding anovulation, tubal disease, endometriosis and impaired semen quantity and quality.

All participants monitored daily luteinizing hormone (LH) levels using ovulation prediction kits (Assure Ovulation Predictor, USA). Endometrial biopsies were timed between 7 and 11 days after the pre-ovulatory LH surge (LH+7 to LH+11). Sample was obtained using a Wallach Endocell<sup>TM</sup> sampler (Wallach, USA) under ultrasound guidance, starting from the uterine fundus and moving downwards to the internal cervical os.

The samples were split into two. One part for the purposes of immunohistochemistry, the endometrial tissues were formalin-fixed for 12-24 hours, paraffin-embedded and serially sectioned (5micronmeters) onto polysine glass slides.

All patient samples were analysed for the uterine NK cell count and then further divided into high and low uNK groups to do further experiments. Only women with previous recurrent miscarriages were used in the experiments. Sixty three patients were recruited and all of them had the uNK cell testing. Table1 below shows the patient characteristics for all the patients. There was higher number of high uNK cell population.

	Control group (n=18)	Study group (n=37)
<b>Age</b>		
Range	29-41	31-40
Median	35	38
<b>Number of miscarriages</b>		
Range	3-5	5-12
Median	4	6
<b>Previous live births (%)</b>	20	0
<b>CD56+ (%)</b>		
Range	1.1-4.5	5.3-27.8
Median	2.3	11.75

**Table 1:** Comparison of Clinical and endometrial characteristics of women in the study- controls, RMC with low uNK cell population and RMC with high uNK cell population

Two-thirds of the biopsy specimens show high uNK cell count whereas literature reports 30% of recurrent miscarriage population show high uNK cell count. This discrepancy is because of inclusion of both recurrent miscarriage and recurrent implantation failure patients. However, the experiments used biopsy specimens only from recurrent miscarriage population.

During the cell culture process, there was significant attrition in the number of samples(n) due to different reasons including insufficient sample size, infection of the cells, insufficient amount of extracted samples for mRNA or for protein analyses. Table 2 shows the patient characteristics recruited for cell culture studies.

<b>uNK cell count</b>	<b>Low &lt;5%</b>	<b>High ≥5%</b>
	(n=12)	(n=10)
<b>uNK cell density (% , mean±SE)</b>	3.2±0.3	10.2±1.3
<b>Age (yrs, mean±SE)</b>	34.3±1.6	36.8±1.7
<b>Past history of previous pregnancy</b>		
<b>Gravida</b>	3 (3-6)	5 (3-9)
<b>Parity</b>	0 (0-1)	0 (0-1)
<b>Miscarriage</b>		
<b>First trimester</b>	3 (0-6)	5 (0-8)
<b>Second trimester</b>	0 (0)	0 (0-3)

**Table 2:** Patient characteristics of women in the cell culture experiments- controls, RMC with low uNK cell population and RMC with high uNK cell population

### 2.2.3 Endometrial samples for primary cell culture

The other part of the biopsy sample was collected in 10% DCC/FCS DMEM/F12 supplemented with antibiotic/antimycotic.

## **2.3 Cell Culture**

### **2.3.1 General maintenance of cell cultures**

All HESCs were managed in a standard cell culture incubation conditions using a NU-5500 Direct Heat Autoflow CO<sub>2</sub> Incubator (NuAire, Inc), which provided a humid atmosphere with 5%v/v CO<sub>2</sub> maintained at 37<sup>0</sup>C. Class II labguard Microbiological Safety Cabinet (NuAire, Inc) was used for cell culture. The working surface of the cabinet was swabbed with 70% alcohol and the cabinet was left running for 5minutes before commencing work. Sterile plastic tissue flasks (Corning), plugged disposable serological pipettes (Corning) and plastic universal tubes (Sterilin) were used for routine culturing.

### **2.3.2 Preparation of Dextran coated charcoal (DCC) stripped fetal calf serum (FBS)**

FBS contains endogenous steroid hormones that may mask the effect of exogenously added ligands. Serum used for cell culture was therefore stripped of small molecules by DCC treatment. A 500ml of FBS had 1.25g of charcoal and 125mg of dextran added and the solution incubated in a water bath at 56<sup>0</sup>C for 2hours, inverting at regular intervals. Following centrifugation at 4000g for 30 min, the supernatant was filter sterilized using a 0.2µm bottle neck filter into a sterile bottle, aliquoted and stored at -20<sup>0</sup>C. For cell culture DMEM/F12 was supplemented with 10, 5, 2 and 0.5%v/v DCC/FCS.

### **2.3.3 Primary human endometrial stromal cell culture**

Samples were collected in Earle's buffered saline containing 100U/ml penicillin and 100 µg/ml streptomycin. The fresh endometrial biopsy was washed twice with 1:1 mixture of serum free DMEM and Ham's F12 (DMEM/F12) to remove mucous and blood. The sample was then finely minced in a Petri dish using scalpels. Following this, the tissue was transferred into a T-25cm<sup>2</sup> flask containing 10ml of digest media which enzymatically dispersed the HESC's. The digestion media consisted of additive free DMEM containing 0.5mg/ml collagenase type IA (Sigma) which disintegrated the extracellular matrix and 0.1mg/ml DNase I (Roche) which eliminated the viscous DNA released during the breakdown process as some cells die. The biopsy sample was left to digest at 37<sup>0</sup>C by vigorous shaking every 20 minutes for the first hour. Following this, 10ml of 10% DCC/DMEM was added to stop the collagenase activity. After centrifugation at 400g for 4min, the pellet containing epithelial, glandular, stromal and red blood cells was resuspended in a maintenance medium of DMEM/F12 containing 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS), 1% L-glutamine, and 1% antibiotic-antimycotic solution.

The cells were then transferred to a T-75cm<sup>2</sup> flask and left undisturbed for an hour at 37<sup>0</sup>C. The stromal cells differentially adhere earlier compared to the epithelial cells thus allowing their separation. Morphologically the stromal cells were examined for their development and confluence every 24 hours.

After 1hr, the media contained epithelial cells and red blood cells which were transferred to a second T-75cm<sup>2</sup> flask. The flask with stromal cells was replenished with fresh 10% DCC/DMEM. This method of separation and the purity has been assessed in our laboratory and is well established {Brosens, 1999, Progesterone receptor regulates decidual prolactin expression in differentiating human endometrial stromal cells}. The proliferating stromal monolayer cells were maintained in 10% DCC/DMEM. Once they reached confluence, they were passaged. The medium was briefly removed, the cells washed with 10ml of pre-warmed PBS and then aspirated. Also 5ml of pre-warmed trypsin-EDTA was added for 5 min at 37<sup>0</sup>C, followed by gentle tapping of the flask. Trypsin activity was then terminated by adding 10ml of 10% DCC/DMEM. The cells were resuspended by centrifuging at 1000rpm for 5min and then cultured at a suitable dilution, typically 1:3. Experiments were carried out in 6 well plates in 10% DCC/DMEM. All experiments were carried at or before the third passage when the cells reached~ 80% confluence.

### **2.3.4 Hormone treatment of cells**

All monolayers were cultured in 2% DCC/DMEM for 24h prior to treatment. All hormone treatments were carried out in 2%DCC/DMEM. In decidualization experiments, confluent monolayers were maintained in DMEM/F12 now containing 2% DCC-FBS and treated with 0.5mM 8-bromoadenosine cAMP(8-bromo-cAMP) (Sigma,St.Louis,MO) alone or in combination with 1 µM medroxyprogesterone acetate (MPA) (Sigma), 1 µM P4 (Sigma), 0.1 µM dexamethasone (DEX) (Sigma), 0.1 µM E (Sigma), 1 µM

aldosterone (Sigma), 10  $\mu$ M RU26752 (Sigma), 0.1  $\mu$ M carbenoxolone disodium salt (CBX) (Sigma), or 0.1  $\mu$ M PF 915275 (PF) (Tocris Bioscience, Abingdon, UK).

### **2.3.5 Transient transfection**

Primary HESCs, cultured in 12-well plates until confluency, were then transfected using the ProFection mammalian transfection kit (Promega, Madison, WI) as per the manufacturer's instructions. Prior to transfection, the media was changed to 1.5ml DMEM/F-12 without antibiotics and supplemented with 5% DCC-FBS until 80-90% confluency. Each well was treated with 100nM of the following small interfering RNA (siRNA) reagents (Dharmacon, Lafayette, CO): si*CONTROL* nontargeting (NT) siRNA pool, GR si*GENOME* SMARTpool siRNA, or MR si*GENOME* SMARTpool siRNA. The cells were incubated at 37°C in a humid atmosphere maintained at 5% CO<sub>2</sub>. After 24h of incubation, the media was replaced and treated as indicated and the cells were harvested when appropriate.

## **2.4 Tissue specimens**

Endometrial biopsies were obtained as detailed earlier in section 2.2.2.



## **2.4.1 Protein Analysis**

### ***2.4.1.1 Protein extraction from cell for Western blot analysis***

Cells were grown in 6-well plates and treated or transfected as appropriate. The cells were washed with ice cold PBS and whole cell extracts were obtained by lysing the adherent cells directly in 100µl of protein lysis RIPA buffer containing protease inhibitors. Cells were scraped, transferred to 1.5ml tubes and stored at -20°C. Protein concentration was determined and typically 30µg of protein combined with protein loading buffer were boiled at 100 °C for 5 min and loaded in SDS-polyacrylamide gels.

Alternatively, protein was extracted using 100µl protein loading Laemmli buffer previously heated to 90°C. Cells were scraped, transferred to 1.5ml tubes, sonicated for a few seconds, boiled for 5 min at 100°C and centrifuged at 15,000g for 5min at 4°C. Equal volumes of each sample were loaded into the gel.

### ***2.4.1.2 Loading buffer (Laemmli buffer)***

Loading buffer has a high detergent content and along with the heat of this extraction protocol, it ensured instant and complete protein denaturation thus preserving any post-translational modifications like sumoylation and phosphorylation. HESCs were cultured in 6-well plates. An equal volume of loading buffer preheated to 85 °C was added to each well, scraped and transferred into microcentrifuge tubes. The lysates were then heated for 5min at 100°C and were snap-frozen on dry ice and stored at -80°C.

#### ***2.4.1.3 Determination of protein concentration***

Quantification of total cell protein concentrations were carried out for Western analysis. Bradford assay reagent containing Coomassie dye was used to determine protein concentration. On binding to arginine and hydrophobic amino acid residues in the protein, this dye exhibits an absorbance shift with the bound form of the dye which is blue showing an absorbance spectrum maximum at 595 nm. The unbound anionic forms are green and red. A set of standards were created using bovine serum albumin (BSA) diluted in distilled water at concentrations 0,1,2,4,8,12,16,20µg/µl with the final volume being 800µl of distilled water. The proteins samples were diluted 2:800 in 800µl and by adding 200 µl of Bradford reagent, a final volume of 1ml was produced for each standard and sample. This sample was mixed well and incubated at room temperature for 30mins. Following this, 200µl of sample and standard was loaded in duplicate into flat bottom optically clear 96-well plate and absorbance read at 595 nm using OPTImax™ microplate reader (Molecular Devices) and analysed using SOFTmax® PRO software (Molecular Devices). The protein concentrations were then calculated by reference to the standards.

#### ***2.4.1.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE)***

Proteins were resolved on discontinuous polyacrylamide gels using Invitrogen Xcell SureLock Mini-cell apparatus. The resolving gels were made

using 10% acrylamide, 1% SDS and 375 mM Tris-HCL pH 8.9. The stacking gels were made using 5% acrylamide, 1% SDS and 125 mM Tris-HCL pH 6.8. Polymerizations of these gels were initiated adding TEMED and APS. The resolving gel poured between the gel plated up to about 2cm from the top and overlaid with saturated butan-2-ol. The butan-2-ol was rinsed off after polymerization was complete and stacking gel was added on top of the resolving gel and appropriate comb inserted. The comb was removed once the stacking gel was set. The gel plate was fitted onto the electrophoresis tank and running buffer (1x) added. An equal amount of protein sample (typically 30 µg) was diluted 1:1 loading buffer and denatured at 100<sup>0</sup>C for 5min. Samples were loaded alongside pre-stained molecular weight markers. Gels were run in 1x Western SDS running buffer at 80V for approximately 30min allowing slow and even entry of the samples into the stacking gel. Subsequently, the gels were resolved at 125 V for 1 hour until the dye had migrated to the bottom of the gel. The gels were then removed from the cassettes for transfer.

#### ***2.4.1.5 Western Blotting***

The Invitrogen Xcell II<sup>TM</sup> Blot Module was used to transfer resolved proteins from gel onto a polyvinylidene fluoride (PVDF). The PVDF transfer membrane was first activated in 100% methanol. The following sequence was created onto the cathode: one sheet of Whatman filter paper, the gel, a PVDF transfer membrane and another piece of Whatman filter paper. This sandwich was rolled to eliminate air bubbles and an anode plate was fitted

on top. A voltage gradient of 25V at 230mA was created perpendicular to the gel for 2h. This allowed migration of the negatively charged particles from the gel towards the anode and then deposited onto the PVDF membrane. The membrane was then air dried and reactivated in 100% methanol. Following this, the membrane was blocked in Western blocking buffer for 30mins at room temperature (RT) and then at 4<sup>0</sup>C overnight on the roller. Following day, the membrane was washed with 1x TBS-T for 5 min. The membrane was then treated with primary antibody diluted in Western primary antibody incubation solution and then incubated for 2h at RT on the roller. After three 15 min washes with 1X TBS-T, the membrane incubated for 1h at RT with HRP-conjugated antibody, raised against the primary antibody, diluted in western secondary antibody incubation solution. After three 15minute washes with 1x TBS-T;, the protein bands were visualised by enhanced chemiluminescence with ECL plus Western blot detection system as per manufacturer's instructions. The membrane was then exposed to autoradiography films.

#### ***2.4.1.6 Stripping of membranes for Western Blotting***

In order to re-probe the PVDF membrane with different primary antibodies, the antibodies were stripped off the membrane with Western stripping buffer at 60 <sup>0</sup>C for 30 min. The membrane then washed with 1X TBS-T three times 10 min each, then blocked and incubated with the new antibody.

## 2.4.2 RNA isolation and extraction

An allocated workbench for RNA decontaminated using RNaseZAP (Ambion) and only RNase-free plastic ware were used for this procedure to minimise the risk of RNA degradation and RNase contamination. All solutions were prepared with diethylpyrocarbonate (DEPC) treated water. Chloroform, isopropanol and 70% v/v ethanol and microcentrifuge tubes were pre-chilled at  $-20^{\circ}\text{C}$  prior to the extraction.

Firstly, total RNA was extracted from the cells using STAT-60 reagent as per the instructions from the manufacturer. STAT-60 is a monophasic solution of phenol and guanidine isothiocyanate that maintains the RNA integrity whilst simultaneously disrupting cells and dissolving cellular components. Cells in 6-well plates were directly lysed by adding 400  $\mu\text{l}$  of the reagent and the cells scraped. The samples were allowed to incubate for 5min at RT to allow complete dissociation of nucleoprotein complexes and then transferred to 1.5ml tubes. To each sample 20% volume of chloroform was added and the microcentrifuge tube vortexed for 15sec and samples stored at  $-80^{\circ}\text{C}$  for a minimum of 30min. The tubes were then centrifuged at 12,000g for 30min at  $4^{\circ}\text{C}$  to separate the solution into a lower red, phenol-chloroform phase, an interphase and a colourless aqueous phase. The RNA remained in the aqueous layer while the DNA and proteins extracted into the organic phase and the interphase. The aqueous top layer was then transferred to a new pre-chilled tube. The RNA was precipitated by adding 50% volume of ice cold isopropanol. Samples were then vortexed and incubated at RT for 10 min and centrifuged at 12,000g for 15min to create a white RNA pellet. The

supernatant was then discarded. The RNA pellet was washed with 1:1 volume of 70% v/v ethanol and centrifuged at 12,000g for 15min. The purified RNA was then air dried, dissolved in a suitable volume of DEPC treated water and stored at -80 °C. RNA concentration was determined using a spectrophotometer at  $A_{260}$  (ND-1000, NanoDrop). Pure RNA shows an  $A_{260}/A_{280}$  ratio of 1.8-2.1 with lower ratios indicating presence of contaminants like proteins and phenol.

## **2.4.3 Gene Expression Analysis using Real Time Quantitative PCR (qRT-PCR)**

### ***2.4.3.1 The Principle***

The measurement of mRNA levels was used to indicate gene expression. This involves removal of genomic DNA from the samples followed by first strand complimentary DNA (cDNA) synthesis and then relative quantification of the target template using specific primer pairs and RTQ-PCR. RTQ-PCR is based on detection of a fluorescent signal produced proportionally to the PCR product during each cycle i.e. in real time, as opposed to the end point detection by conventional quantitative PCR methods. Although several detection chemistries can be used, the simplest and economical is SYBR green, a dye that binds all double stranded DNA molecules. Upon binding, it emits fluorescence in proportion to the double stranded amplicons. The laser light emitted via optical fibres leads to excitation of the fluorophores within the samples and the resulting fluorescence signal is directed to a spectrograph attached to a charged coupled device camera detector. The

SYBR green assay requires optimisation as it can detect non-specific PCR products like primer-dimers.

The ABI StepONE sequence detection system (Applied Biosystems) software calculates the change in fluorescence intensity ( $\Delta R_n$ ) due to amplification. This is calculated using the formula  $\Delta R_n = R_n^+ - R_n^-$ , where  $R_n^+$  is the fluorescence emission of the product at each time point and  $R_n^-$  is the fluorescence emission of baseline usually between 3-15 cycles. The software further constructs amplification plots of  $\Delta R_n$  vs cycle number. As the amplification proceeds, the change in fluorescence intensity reaches a threshold, which is defined as a statistically significant point above the baseline, usually determined as 10 times the standard deviation of the baseline (cycles 3-15). The threshold cycle ( $C_t$ ) is calculated by determining the point at which the fluorescence crosses the chosen threshold limit that correlates to the initial amount of target quantity.  $C_t$  values decrease linearly with increasing input target quantity.

#### ***2.4.3.2 Primer selection and Optimisation***

The length and location of PCR primers were chosen using the Primer 3 Output Program (<http://frodo.wi.mit.edu>). Primers were selected on different exon or intron-exon boundaries to avoid contaminating genomic DNA. To reduce the possibility of amplifying genomic DNA not complimentary from the sample; the primer pairs were selected within a short sequence of DNA crossing an intron-exon boundary. All primer pairs were optimised with annealing temperatures around 60°C. Lyophilized primers were diluted in

DEPC-treated water to storage concentration of 100pmol/μl and later diluted again to a working concentration of 20pmol/μl.

Each primer pair was optimised using a combination of forward and reverse primer concentrations ranging from 50nM to 900nM using a fixed amount of cDNA template. Optimal primer concentration was chosen based on the lowest Ct and highest ΔRn. Primer details are as seen in Appendix II.

#### **2.4.4 Isolation of genomic DNA and cDNA synthesis**

In order to remove small quantities of genomic DNA contamination, DNase I, Amplification grade from Invitrogen was used. One Unit of DNase I was added to 1μg of total RNA and incubated for 15min at RT in the presence of 1 x DNase I reaction buffer in a total volume of 10μl made up with DEPC-treated water. The reaction was stopped by adding 0.5μl 25mM EDTA incubating at 65<sup>0</sup>C for 15min.

SuperScript<sup>TM</sup> First Strand Synthesis System for RT-PCR kit from Invitrogen was used to generate complimentary deoxyribonucleic acid (cDNA) from total RNA. Eight μl of DNase I treated RNA was mixed with 1μl deoxyribonucleotide triphosphate (dNTP) mix (10mM of each dATP, dTTP, dGTP, dCTP) and 1μl Oligo (dT) (0.5μg/μl) and the volume made up to 10 μl with DEPC-treated water. Samples were incubated at 65<sup>0</sup>C for 5min and placed on ice for 1min. A master mix (for n+1samples) was prepared consisting of 2μl 10x RT buffer, 4μl 25mM MgCl<sub>2</sub>, 2μl 0.1 M DTT and 1μl RNase OUT (40U/μl). Nine μl of mastermix was added to each RNA/primer



mix and samples collected by centrifugation and incubation at 42<sup>0</sup>C for 2min. One µl (50U) SuperScript II Reverse Transcriptase was then added to each reaction and samples were incubated for a further 50min at 42<sup>0</sup>C. The reactions were then terminated by incubation at 70<sup>0</sup>C for 15min. The residual RNA template from the newly synthesised cDNA-RNA hybrid molecule was digested using 1µl RNase H (2U) for 20min at 37 <sup>0</sup>C. cDNA samples were stored at -20 <sup>0</sup>C.

### **2.4.5 Amplification of Target cDNA**

The mRNA detection was done using SYBR green. All reactions were set up in a 96-well plate as follows: 1µl cDNA template, 12.5µl SYBR green mastermix and an appropriate ratio of primers in a total volume of 25µl made up with DEPC-treated water. Negative controls without cDNA template were included. All measurements were done in triplicate in the ABI StepONE Sequence Detection System (Applied Biosystems). The plate was then heated at 95<sup>0</sup>C for 10min to activate the AmpliTaq Gold DNA polymerase. The amplification process then took, 40 cycles of 95<sup>0</sup>C for 15sec (denaturation), 60<sup>0</sup>C for 1min (annealing and extension). Data analysis was carried out using the  $\Delta\Delta$ -Ct method. RNA input variances were normalized against the levels of the L19 housekeeping gene, which encodes a ribosomal protein.

## 2.5 Microarray analysis

Genome-wide microarray analysis was performed on primary cultures established from four different patients. Each culture was first transfected either with NT-siRNA or siRNA oligos against MR or GR. The cells were then decidualized for 4 days with 8-bromo-cAMP, Progesterone, and E (Cortisone). Total RNA was extracted using STAT-60 reagent (AMS Biotechnology, Abingdon, UK). RTQ-PCR was performed after first-strand cDNA synthesis to determine the level of GR and MR knockdown. RNA quality was analyzed on an Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany). Microarray analysis on total

RNA samples was performed by UCL Genomics using Bioconductor version 2.0 and R version 2.9.0. The robust multi-array analysis algorithm was used to obtain normalized data and gene signals. This method performs within-chip and between-chip normalizations in a single step. Gene summaries are generated using the Affymetrix Expression Console software. The criteria used to generate lists of differentially expressed genes are based on standard filtering by fold change (50%change) and include false discovery rate filter ( $p=0.05$ ). Gene ontology annotation was performed using the Database for Annotation, Visualization, and Integrated Discovery Bioinformatics Resources 6.7 (SAIC-Frederick, Inc., Frederick, MD). Microarray data have been deposited in Gene expression omnibus (GEO) database, accession number GSE42538.

## **2.6 Enzyme-linked immunosorbent assay (ELISA)**

ELISA is a biochemical technique used to detect the presence of an antibody or an antigen in a sample. Quantikine Human IL-11 and IL-15 immunoassays (R&D Systems) were used and performed as per the manufacturer's protocol.

Principle: The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-11/ IL-15 was precoated onto the 96 well polystyrene microplate. Standards and samples were pipetted into the wells and any IL-11/IL-15 present was bound to the immobilised antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for IL-11/IL-15 was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. Subsequently, a colour develops in proportion to the IL-11/IL-15 bound in the initial step. The colour development is stopped and the intensity of the colour measured.

Procedure: The supernatant from the cultured and treated HESCs were stored at -20°C. One hundred µl of assay diluent was added to each well. Fifty µl of standard/sample/control was then added per well and covered with adhesive strip. This was incubated for 3 hours at RT. Each well aspirated and washed with 400µl wash buffer, repeating the process 3 times for a total of 4 washes. After the last wash, the plate is inverted and blotted. Two hundred µl of the conjugate (IL-15/IL-11) was added to each well and plate covered with a new adhesive strip and incubated for 45 min at RT. The

process of washing and aspirating repeated. Subsequently, 200µl of substrate solution were then added to each well and incubated at RT for 30 min, protected from light. Finally, 50µl of stop solution added to each well which changes the colour of the solution from blue to yellow. The optical density of each well determined within 30min using a microplate reader set to 450nm. The wavelength correction was set to 540 nm to 570 nm.

## **2.7 Confocal immunofluorescence microscopy**

Primary HESCs cultured on glass slides were fixed with 4% paraformaldehyde (Sigma) and permeabilized by 0.1% Triton X-100 (BDH Chemicals, London, UK). BODIPY 493/503 (Invitrogen Ltd.) was applied at 1 µg/ml in PBS. BODIPY-stained samples were washed with PBS thrice for 5 min before imaging. 4,6-Diamidino-2-phenylindole (DAPI) was used to identify nuclei. We examined samples under epifluorescent optics, and digital image were obtained with a Zeiss 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

## **2.8 Immunohistochemistry (IHC)**

The endometrial biopsy tissue was fixed in neutral buffered formalin overnight at RT. IHC was performed to stain for the CD56 bright uterine NK cells. IHC was used to categorise the samples into high and low uterine NK cell population. The subjective nature of assessment in IHC has been its major drawback as an analytical technique. This was overcome by using

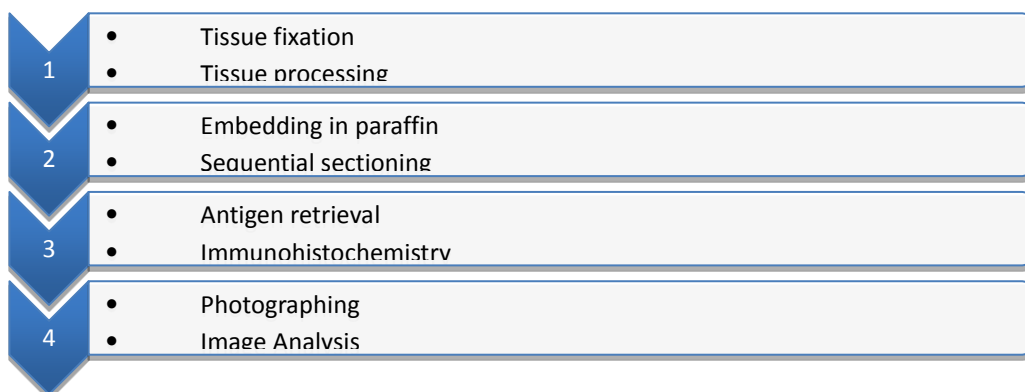
ImageJ analysis which is a semi-automated digital image which produces highly reproducible results for stromal cell counting with the use of the point picker tool for uNK cell counting {Drury, 2011, Endometrial cell counts in recurrent miscarriage: a comparison of counting methods}.

## 2.8.1 Principle

Immunohistochemistry staining technique allows the visualisation of the antigens through sequential application of a specific antibody to the antigen (primary antibody), a secondary antibody to the primary antibody and an enzyme complex with a chromogenic substrate with interposed washing steps. The enzymatic activation of the chromogen results in a visible reaction product at the antigen site. The specimen is then counterstained and cover slipped. The slide is then scanned and analysed.

## 2.8.2 Procedure

The procedure was performed with the help of research histopathologist Sean James.



**Figure 2.1:** Schematic process from tissue sampling to analysis

Adhesive coated slides were pre-labelled, one slide for each antibody, plus several spares for negative controls. Sections are cut at 4  $\mu\text{m}$  and dried overnight in the 56  $^{\circ}\text{C}$  oven.

*Antigen retrieval using PickCell Antigen retrieval unit:* For antigen retrieval the slides incubated for 30min at 50 $^{\circ}\text{C}$  in 1mM EDTA, pH 8.0. Seven ml of Tris-EDTA buffer concentrate at pH7.8 or buffer A (pH6), B (pH8), C (pH4.5) or U (pH6) and 63 ml distilled water was placed into a slide chamber in the antigen retrieval unit. To optimise the antibody, tissues were exposed to different pH buffers to find the best one. The slides are inserted into the diluted buffer. The lid was closed and left for 2h. During this time, the unit reaches the temperature/pressure required and then cools down to a safe level for handling.

*Optimisation:* Sufficient numbers of known positive control sections of 3 $\mu\text{m}$  each were cut freshly (to avoid reduction in epitope activity). The slides are then exposed to a range of pH's in the PickCell antigen retrieval unit – pH4.5, pH6.9, pH7.8, pH8.0. Subsequently, they were stained (as described below) at a range of serial dilutions based on the manufacturer's recommended dilutions- 1/50, 1/100, 1/200, 1/400, 1/800, 1/1600. Following staining, the slides were checked microscopically and a judgement was made to choose the best dilution based on the level of background staining and the level of non-specific staining seen.

*Staining technique:* Following antigen retrieval, the sections were washed with distilled water. The borders around the sections marked with a hydrophobic pen and the slide placed in a metal moist chamber.

Endogenous peroxidase activity was blocked with Leica peroxidase block ensuring the tissue on each slide is covered with the solution and left for 5min. The moist chamber was covered after each step. This was followed by two washes with TBS for 5min each time. Then, the tissue was covered and incubated with 100µl Leica protein block, incubated for 5min and the solution drained off. Subsequently, the slide was incubated in 200µl of primary antibody (optimal dilution determined by serial dilutions of known positive controls at varying pH buffers) overnight at 4 °C. The following morning, the slides were washed with TBS for 10min, twice. The slides were then incubated with 100µl of Leica post primary block for 30min at RT. The slides were then washed with TBS twice, 5min each time. Subsequently, they were incubated with 100µl of Leica Novolink polymer solution for 30min at RT. Again, two washes 5min each with TBS performed. The slides now were incubated with 2-3 drops of Leica DAB working solution (50µl of DAB chromogen in 1ml of Novolink DAB substrate) and left for 5min. The slides washed twice, 5min each with TBS and drained off. The slides were then counterstained with haematoxylin for 1min, washed in distilled water followed by incubation in TBS for 2-3 min to blue haematoxylin. The slides were then placed into a metal slide rack, dehydrated and mounted.

### **2.8.3 Construction of Tissue Micro Array (TMA)**

Areas of interest, i.e. subepithelial regions, were spotted and tissue microarrays comprising duplicate 0.6mm cores from 18 cases in each group were constructed using Alphelys TMA Designer R 2 version 1.0.0.8. Sections

(3µm) were cut from completed array blocks and transferred to silanized glass slides. Sections from these arrays were then stained for CD56, 1:200 (NCL-CD56-1B6; Novocastra), 11βHSD1, 1:300 (AB83522; Abcam, Cambridge, UK), MR, 1:400 (H-300: SC-11412; Santa Cruz Biotechnology, CA) and GR, 1:200 (E-20: SC-1003; Santa Cruz). Semi-quantitative analysis was performed using imageJ software and the color deconvolution plug-in described below.

## **2.8.4 Image J Analysis**

'Image J' is a freely available java-based (hence platform-independent) public domain image processing and analysis program developed at the National Institutes of Health, Bethesda, Maryland, USA which is rapidly gaining popularity as an objective, reproducible, cost-effective and time-saving method of automated image- based immunohistochemistry evaluation {Girish, 2004, Affordable image analysis using NIH Image/ImageJ}. It can display, edit, analyze, process, save and print 8-bit, 16-bit and 32-bit images. It can read many image formats including TIFF, GIF, JPEG, BMP, DICOM, FITS and 'raw'. It can calculate area and pixel value statistics of user-defined selections.

Digital images of IHC-stained slides were obtained using Mirax Midi slide scanner. This instrument scans brightfield sections at a x20 objective, at a resolution of 0.23µm/pixel. This produces images that can be dynamically manipulated within the viewer software (Panoramic Viewer from 3D Histech)



this allows optical magnifications up to x20, and digital magnification to x200. Images were saved in bitmap format which was further analysed using the Image J software. Where the epithelial edge was not visualised, they were excluded from the study.

The field of view under x400 under high power is 450 micrometers. The field size is inversely proportional to the magnification. This means is that the field size divided by the same amount as the magnification was multiplied by, gives the field of view. High power field at x40 magnification covers a field view of 4500 micrometers. Five HPF at x40 was taken to analyse the density of CD56+ cells.

#### ***2.8.4.1 Point Picker plug-in***

The point picker plugin available in ImageJ freeware (National Institutes of Health, Bethesda, MS, USA); allows cells to be marked with a coloured cross by clicking with the mouse. Upon completion, the number of counted cells is displayed in a text box. This makes this method the gold standard for cell counting, as cells are marked in the display screen and several observers can comment/discuss and come to an agreement {Drury, 2011, Endometrial cell counts in recurrent miscarriage: a comparison of counting methods}. A grid was applied to each image and the point picker plugin used to quantitate stromal cell number and uNK cell number. As uNK cell density varies with endometrial depth, counting of CD56+ cells was confined to the stroma underlying the luminal epithelium. Five randomly selected high-power fields were assessed per biopsy using imageJ analysis to minimize inter-observer variability {Drury, 2011, Endometrial cell counts in recurrent miscarriage: a

comparison of counting methods;Girish, 2004, Affordable image analysis using NIH Image/ImageJ;Mariee, 2012, The observer and cycle-to-cycle variability in the measurement of uterine natural killer cells by immunohistochemistry}. The uNK cell count is calculated by:

$$\%uNK = \frac{\text{uNK cell count} \times 100}{\text{Stromal cell count}}$$

Normal uNK cell density was defined as 5% CD56 cells in the stroma underlying the luminal epithelium {Quenby, 2005, Prednisolone reduces preconceptual endometrial natural killer cells in women with recurrent miscarriage;Tang, 2009, Prednisolone Trial: Study protocol for a randomised controlled trial of prednisolone for women with idiopathic recurrent miscarriage and raised levels of uterine natural killer (uNK) cells in the endometrium}.

#### ***2.8.4.2 Colour Deconvolution plug-in***

The colour deconvolution plugin for ImageJ implements stain separation was performed using Ruifrok and Johnston's method {Ruifrok, 2001, Quantification of histochemical staining by color deconvolution}. The command Image>Color>RGB split, this plugin unmixes an RGB (Red Green Blue) image produced by subtractive mixing (inks, histological dyes) into separate channels corresponding to up to 3 determined colors. This is useful e.g. to do pigment separation. The plugin provides a number of "built in" stain vectors; the ones used in the study include Haematoxylin and Eosin (H&E) and Haematoxylin and DAB (H DAB). This tool was used in the analysis of immunostained intensity to evaluate antigen expression. The antigen expressions analysed are 11 $\beta$ HSD 1, GR and MR. Each core in the TMA was separately analysed and represented as percentage area of positively stained (H DAB) area compared to the stroma (H&E).

## **2.9 Radio-thin layer chromatography**

The activity of 11 $\beta$ HSD1 in HESCs was analyzed essentially as described in literature {Su, 2004, Novel 18beta-glycyrrhetic acid analogues as potent and selective inhibitors of 11beta-hydroxysteroid dehydrogenases}. Briefly, the microsome-containing fraction of undifferentiated and decidualized HESCs was incubated with reduced nicotinamide adenine dinucleotide phosphate (NADPH) (1mM) and [3H] E. [14C] F (3X10<sup>3</sup>dpm) was added to monitor procedural losses together with 50 $\mu$ g of unlabeled F. The assay was performed under initial rate conditions, and less than 12% of the substrate was converted to product. Precursor and product steroids were separated by thin layer chromatography using chloroform:methanol (9:1, vol/vol).

Retention factor values for F and E were 0.5 and 0.8, respectively. The amount of radioactivity was measured by scintillation spectrometry. Enzyme activity was determined as the amount of product formed per milligram of protein per hour after correction for procedural losses.

## **2.10 Statistical analysis**

Data was analysed using the statistical package Graphpad Prism version **6.0** (Graphpad software Inc, San Diego, CA, USA). Student's *t*-test and Mann-Whitney U test were used when appropriate.

Logarithmic transformations were used when data were not normally distributed. This was particularly used for gene expressions as gene expression levels are skewed in linear scale. This is because some of the data points (lower expressed genes) are between 0 and 1 where 1 means no change and other data points (higher expressed genes) are between 1 and positive infinity. Log transformation also made the data more symmetrical and the parametric statistical test becomes more accurate.

Variables that were not normally distributed were analysed using Mann-Whitney *U* test for paired comparisons. This was used for analysing areas of staining were expressed in colour deconvolution. Results were expressed at standard deviations (SD) or means  $\pm$  standard error of the mean (SEM). Statistical significance was assumed when  $p < 0.05$ .

# **Chapter 3: Induction of 11 $\beta$ HSD 1 and Activation of Distinct Mineralocorticoid Receptor and Glucocorticoid Receptor Dependent Gene Networks in Decidualizing**

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## 3.0 Human Endometrial Stromal Cells

### 3.1 Introduction

Glucocorticoid treatment has been advocated as a treatment to improve reproductive outcome for a number of reasons:

1. High levels of uterine natural killer cells have been associated with recurrent miscarriage and recurrent implantation failure, reduction of these occurred with prednisolone.
2. Prednisolone was associated with an improvement in clinical outcome in a pilot trial in women with recurrent miscarriage (Quenby *et al.*, 2005b).
3. Steroids were associated with improvement in IVF outcome in a meta-analysis treatment (Boomsma *et al.*, 2007).
4. Glucocorticoid stimulated peri-implantation hCG secretion from trophoblast of early human embryo (Ringler *et al.*, 1989),
5. Glucocorticoids accelerated trophoblast growth and invasion (Mandl *et al.*, 2006).

However, successful implantation requires some inflammatory events with pro-inflammatory cytokines and prostaglandins (Chard, 1995; Sharkey, 1998; Kelly *et al.*, 2001; Bazer *et al.*, 2010). Glucocorticoid may act to disable the cytokine-prostaglandin signalling cascade for implantation and decidualization because of anti-inflammatory action. Hence is it unclear as to whether glucocorticoids are beneficial to implantation.

Glucocorticoids include active glucocorticoid, cortisol (Kendall's compound, F) and inert glucocorticoid, cortisone (Kendall's compound, E). In glucocorticoid metabolism, the enzymatic reversible inter-conversion of cortisol and cortisone is catalyzed by 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD) family. NADP(H)-dependent enzyme, 11 $\beta$  HSD type1 (11 $\beta$ HSD1) basically regenerates cortisol from cortisone and NAD(H)-dependent 11 $\beta$  HSD type2 (11 $\beta$ HSD2) acts as a dehydrogenase converting cortisol to cortisone (cortisol-cortisone inter-conversion) (Lakshmi & Monder, 1988). The main binding receptor for cortisol is glucocorticoid receptor (GR; NR3C1), but cortisol also has a high affinity for mineralocorticoid receptor (MR; NR3C2), since MR has structural and functional kinship with GR. Therefore excess cortisol binding MR can result in apparent mineralocorticoid excess, such as Cushing syndrome (Arriza *et al.*, 1987). Further, 11 $\beta$ HSD1 is normally found co-localised with GR, while 11 $\beta$ HSD2 is commonly found in MR-expressing tissues. However in adipose tissue, cortisol inactivating enzyme, 11 $\beta$ HSD2 is not significantly expressed, thus cortisol also acts through MR (Yang *et al.*, 1997; Engeli *et al.*, 2004). And the balance between GR and MR assumes the important role for regulation of inflammatory adipocyte responses (Hoppmann *et al.*, 2010).

In this thesis the critical determinant of implantation is considered to be decidualization (chapter 1). Decidualization which is progesterone (P4) dependant process seems to bestow unique functions on the endometrium that are essential for pregnancy, including the ability to regulate trophoblast invasion, to modulate local angiogenesis, to recruit specialized uterine natural killer cells and macrophages, and to resist environmental and

oxidative stress (13, 185). Furthermore in this thesis decidual changes in human endometrial stromal cells (HESCs) are thought to be the most significant component of decidualization (chapter 1).

HESCs express 11 $\beta$ HSD1 and GR predominantly in menses phase, and 11 $\beta$ HSD2 and MR in secretory phase. Hence, both the isoforms of 11 $\beta$ HSD, as well as GR and MR are expressed in human endometrium (McDonald, 2006). In decidualizing endometrium, 11 $\beta$ HSD1, GR and MR, but not 11 $\beta$ HSD2, are expressed (McDonald *et al.*, 2006), similar to adipose tissue. Interestingly, GR expression is confined to stromal cells, whereas MR is reportedly present in both stromal and glandular compartments. Furthermore, 11 $\beta$ HSD1, but not 11 $\beta$ HSD2, is highly up-regulated upon differentiation of primary human endometrial stromal cells (HESCs) into specialist decidual cells *in vitro* (Takano, 2007).

However the role of glucocorticoids via 11 $\beta$ HSD1/GR/MR signalling in the decidual transformation of endometrial stromal cells remains poorly understood. Moreover, GR and MR knockout animals die after birth (Cole *et al.*, 1995; Berger *et al.*, 1998), therefore little is still known about the reproductive consequences of GR and MR absence.

In this chapter we investigated the hypothesis that during decidualization of HESCs the induction of 11 $\beta$ HSD1 leads to increased F bioavailability, which in turn regulates the expression of distinct GR- and MR-dependent gene networks.



## 3.2 Results

### 3.2.1 Expression patterns of 11 $\beta$ HSD1 and 2, GR, and MR in undifferentiated and decidualized HESCs & impact of progesterone and dexamethasone on this signalling pathway

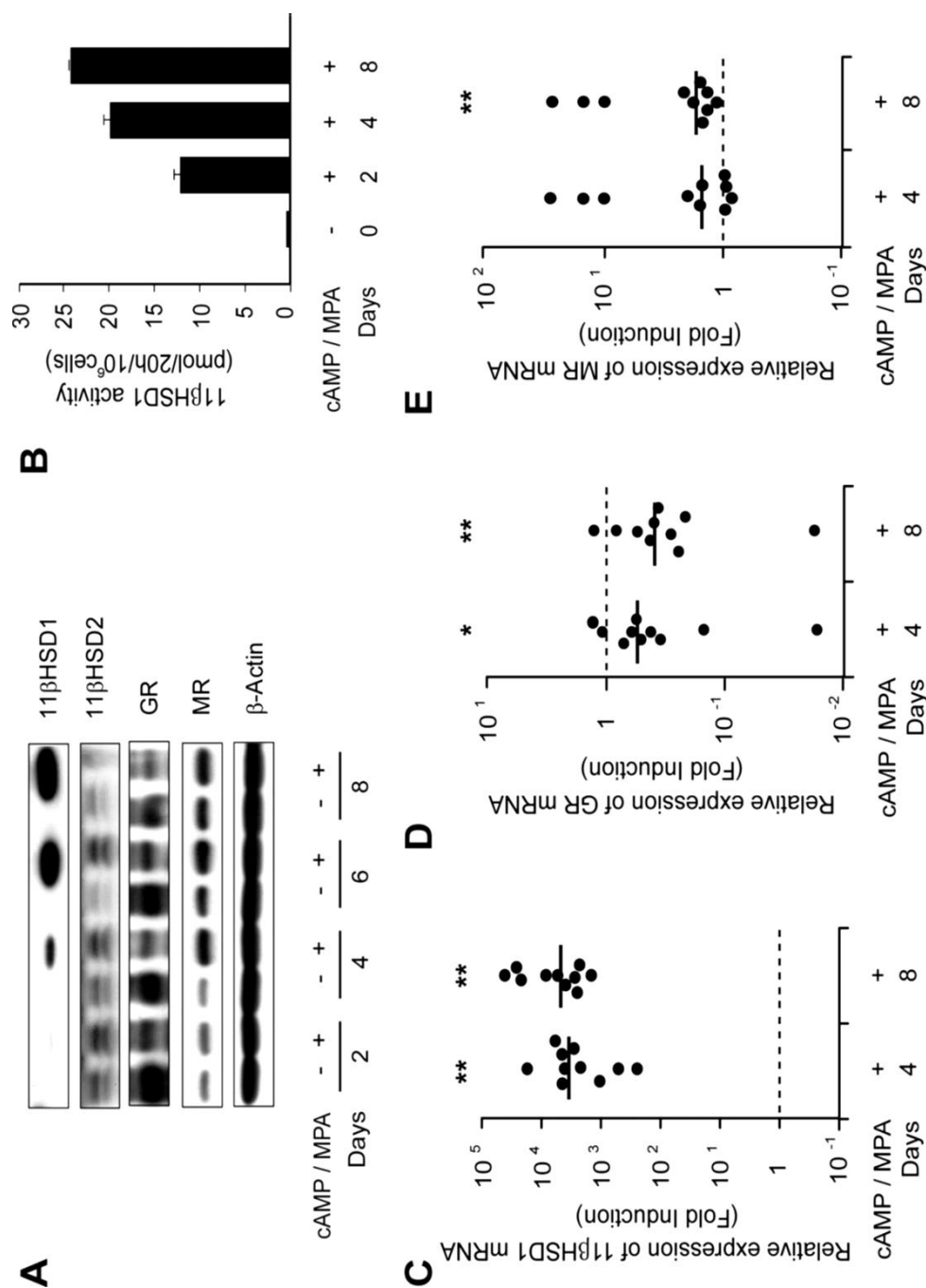
The first experiment was set up to examine the role of corticosteroid signalling in decidualization. Hence to explore the role of 11 $\beta$ HSD1, GR and MR upon decidual change, primary HESCs were prepared as untreated (0 days) or treated with 8-bromo-cAMP and MPA for 2, 4, 6 and 8 days.

Decidualization was determined based on the morphological appearance and changes in the stromal cells. This comprises of change in spindle shaped cells to more rounded cells and the cells become more confluent. Strikingly, the 11 $\beta$ HSD1 mRNA and protein levels were induced significantly upon decidualization as seen in figure 3.1.A. This significant increase in the induction of 11 $\beta$ HSD1 is seen in a time dependant manner, increasing with the days of decidualization. Upon studying the activity of 11 $\beta$ HSD1 decidualizing HESCs using chromatography, the 11 $\beta$ HSD1 activity was upregulated as well as its expression levels as seen in figure 3.1.B. However, the induction of 11 $\beta$ HSD2 expression was low in HESCs, and in the absence of treatment with 8-bromo-cAMP and MPA, expression further declined in response to low-serum (2% DCC-FBS) culture conditions.

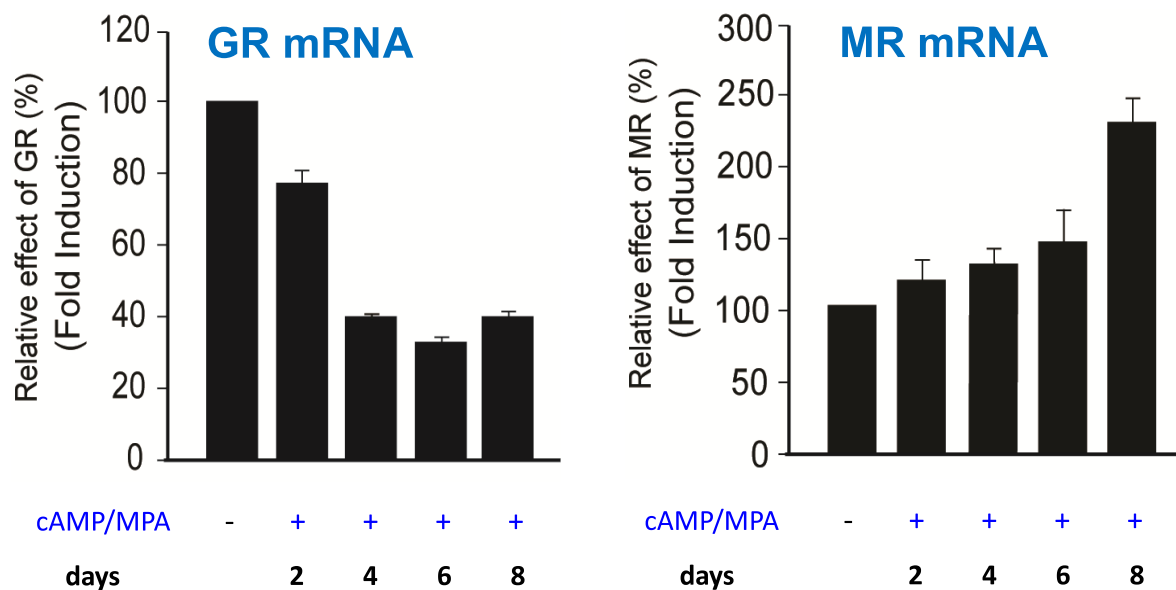
On examining the regulation of 11 $\beta$ HSD1 mRNA in response to differentiation signals in 10 independent primary cultures, the 11 $\beta$ HSD1 transcript levels invariably increased by several orders of magnitude in response to 8-bromo-cAMP and MPA treatment as shown in Fig. 3.1.C.

Undifferentiated HESCs also abundantly express GR. However, treatment with 8-bromo-cAMP and MPA down-regulated this nuclear receptor as seen in Fig. 3.1.A. MR followed a reversed pattern with expression gradually rising in differentiating HESCs. Notably, culturing HESCs in low-serum conditions for several days was sufficient to alter MR and GR expression in opposite directions, although the effect was much more pronounced upon 8-bromo-cAMP and MPA treatment (Fig. 3.1.A). Furthermore, the down-regulation of GR and reciprocal induction of MR at protein level in decidualizing cells were reflected at transcript level (Fig. 3.1., D and E).

The switch from GR dominance to MR dominance is even better appreciated in the bar graph shown in Fig 3.2.



**FIG.3.1.** Expression of 11βHSD enzymes, GR, and MR in deciduolizing HESCs. A, Primary HESCs were treated with 8-bromo-cAMP and MPA in time-course experiments lasting 8 d. Total protein lysates were harvested at the indicated time points and subjected to Western blot analysis for 11βHSD1, 11βHSD2, GR, and MR. β-Actin served as a loading control. B, 11βHSD1 activity was measured using radio-thin layer chromatography in HESCs treated with 8-bromo-cAMP and MPA for the indicated time points. C, Induction of 11βHSD1 mRNA in 10 independent primary cultures treated with 8-bromo-cAMP and MPA for either 4 or 8 d. The data show fold change in expression relative to levels in undifferentiated HESCs (dotted line). Note the logarithmic y-axis. The horizontal bar indicates median. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ . D and E, The same sample set was analyzed for GR and MR, respectively.



**FIG 3.2.** This figure further reinforces the clear switch from GR (glucocorticoid) to MR (mineralocorticoid) dominance at mRNA transcript levels

Medroxy progesterone acetate which was used in the above experiment has both progestogenic and corticosteroid actions, hence the next experiment set up was to study the effect of Progesterone (P4) and dexamethasone (DEX) on their own upon 11 $\beta$ HSD1/GR/MR signalling pathway in decidualizing endometrial cells.

### **3.2.2 P4 drives the expression of 11 $\beta$ HSD1 in decidualizing HESCs**

In order to investigate the effect of progesterone (P4) and dexamethasone (DEX) on the 11 $\beta$ HSD1/GR/MR signalling pathway in decidualizing endometrial cells, cultures were untreated or treated with 8-bromo-cAMP alone or in combination with P4 or DEX for 2,4 and 8 days.

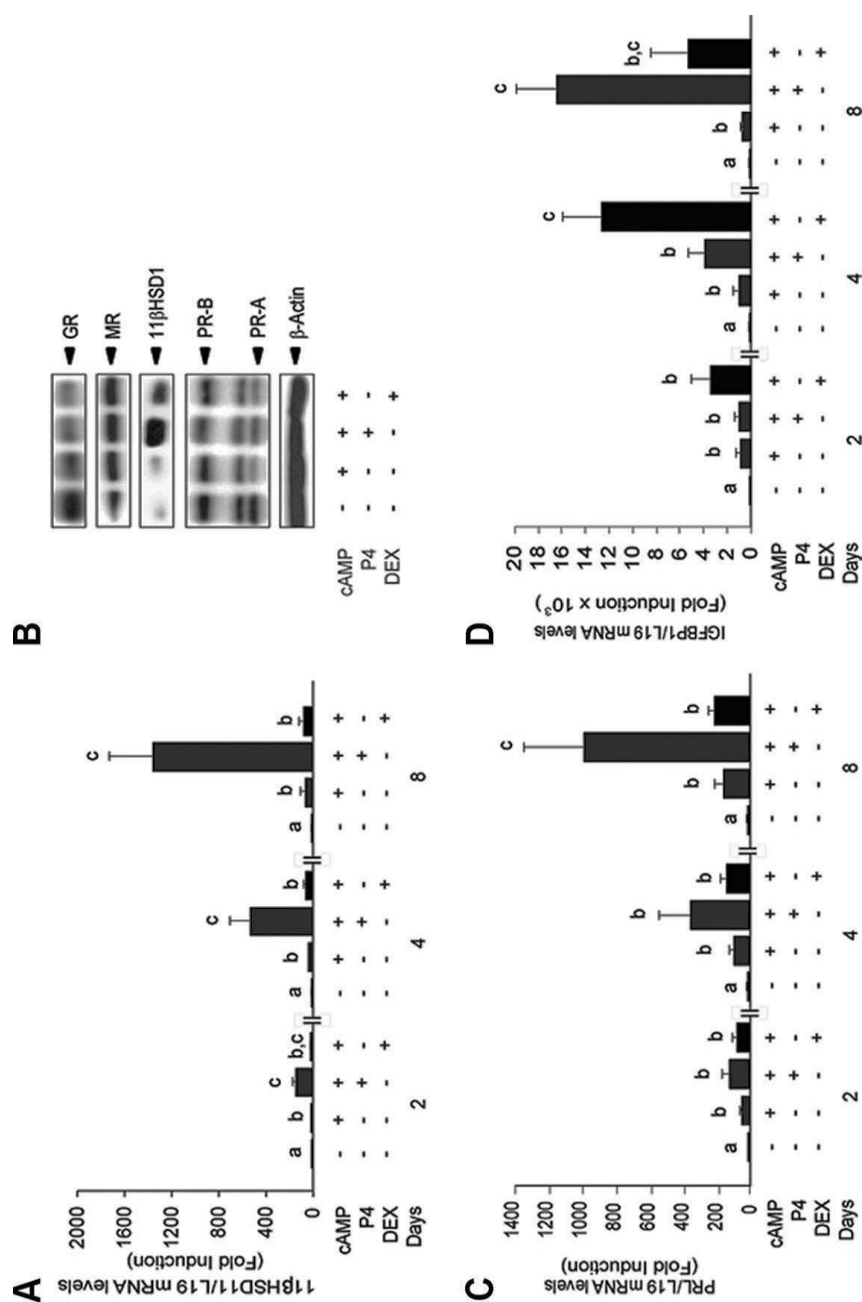
The induction of 11 $\beta$ HSD1mRNA level was undoubtedly dependent on the combination of cAMP and P4 (Fig. 3.3.A). This finding was also reflected in the protein analysis as seen in Fig.3.3.B).

GR, MR, 11 $\beta$ HSD1 and progesterone receptor (PR:NR3C3) belong to a subgroup of nuclear receptors and the steroid receptor superfamily. Thus GR, MR, 11 $\beta$ HSD1 and PR protein levels were confirmed with the parallel treatment for 4 days. GR protein level was reduced by treatment with 8-bromo-cAMP plus P4 or DEX. The 11 $\beta$ HSD1 level was undoubtedly increased by the treatment with 8-bromo-cAMP plus P4, on equality with RTQ-PCR data, but the ability of DEX was insufficient to regulate 11 $\beta$ HSD1.

Time-course analysis demonstrated that, in contrast to P4, DEX had little or no effect on the induction of 11 $\beta$ HSD1 transcripts in 8-bromo-cAMP-treated cells (Fig. 3.3A). Compared with treatment with 8-bromo-cAMP alone, addition of DEX did up-regulate 11 $\beta$ HSD1 protein levels, although this response was much more pronounced with 8-bromo-cAMP and P4 (Fig.3.3B). Western blot analysis also revealed that cAMP signalling drives the down-regulation of GR and reciprocal induction of MR in decidualizing cells (Fig. 3.3B). As previously reported {Brosens, 2009, A role for

menstruation in preconditioning the uterus for successful pregnancy}, 8-bromo-cAMP reduces cellular levels of PR isoforms (PR-A and PR-B). Although co-treatment with P4 induces a mobility shift in PR migration on SDS-PAGE, reflecting ligand-dependent receptor phosphorylation, DEX had no effect (Fig.3.3B).

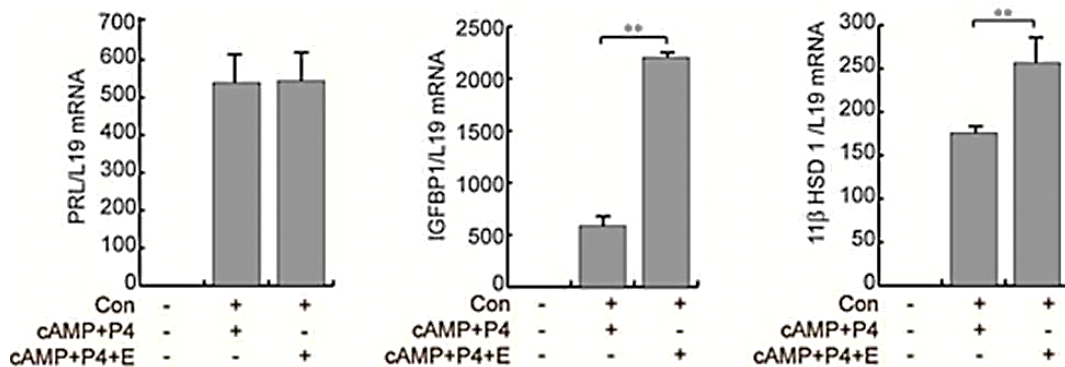
Upon comparing the induction of two decidual markers, PRL and IGFBP1, in primary cultures with 8-bromo-cAMP and either P4 or DEX; unlike P4, DEX had no effect on cAMP-dependent induction of PRL transcripts in HESCs (Fig. 3.3C). However, DEX was more potent than P4 in enhancing IGFBP1 expression in cultures stimulated with 8-bromo-cAMP, especially during the early stages of the decidual process (Fig.3.3D).



**FIG. 3.3** Glucocorticoids selectively modulate the expression of decidual marker genes. A, RTQ-PCR analysis of 11 $\beta$ HSD1 transcript levels in HESCs treated with 8-bromo-cAMP with or without P4 or DEX for 2, 4, and 8 d. The results show the fold change (mean $\pm$ SEM) in 11 $\beta$ HSD1 transcript levels relative to vehicle control of four independent primary cultures. B, Western blot analysis of GR, MR, 11 $\beta$ HSD1, and PR proteins in whole-cell lysates obtained from primary HESCs treated with a combination of 8-bromo-cAMP with or without P4 or DEX for 4 d.  $\beta$ Actin served as a loading control. C and D, Fold change (mean $\pm$ SEM) in PRL and IGFBP1 mRNA levels, respectively, relative to vehicle control of four independent primary cultures. Different letters above the error bars indicate that those groups are significantly different from each other at  $P < 0.05$ .

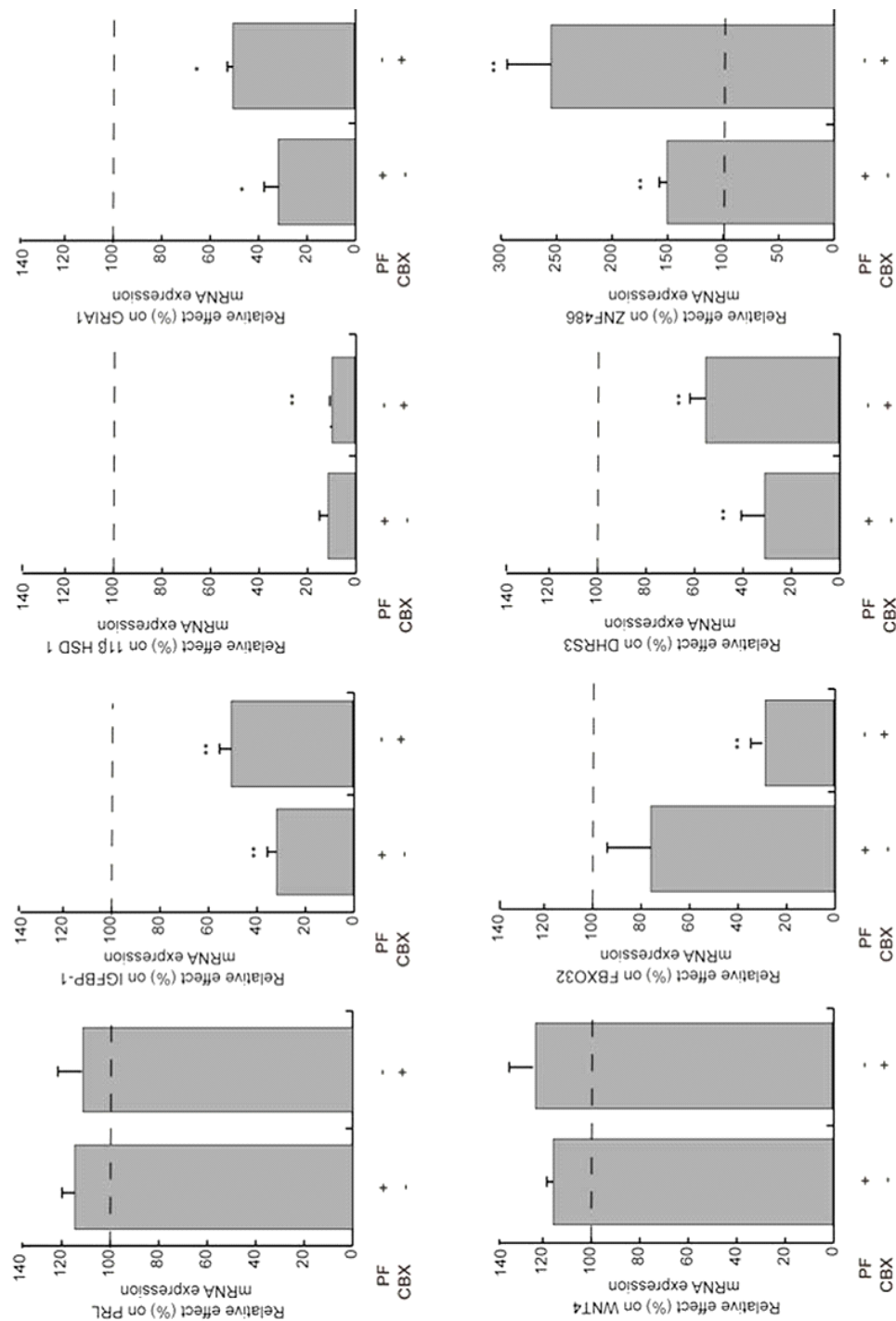
To test whether 11 $\beta$ HSD1-dependent F biosynthesis modulates the expression of decidual marker genes, primary HESCs were decidualized with 8-bromo-cAMP and P4 in the presence or absence of E. The primary cell cultures were differentiated with 8-bromo-cAMP and P4 in the presence or absence of E. After 4 days, the mRNA transcript levels of PRL, IGFBP1 and 11 $\beta$ HSD1 were determined.

Addition of E has no effect on the induction of *PRL*. However, it strongly enhanced the expression of *IGFBP1* and, albeit less pronounced, *11 $\beta$ HSD1* transcripts (Fig. 3.4). Conversely, treatment of primary cultures with an 11 $\beta$ HSD1inhibitor (CBX or PF) attenuated the induction of *IGFBP1* and *HSD11B1* but not *PRL* (Fig. 3.5). These observations underscore that 11 $\beta$ HSD1-dependent cortisol biosynthesis impacts selectively on the expression of decidual marker genes.



**Figure 3.4:** Effect of cortisone (E) on the expression of decidual marker genes. Primary HESCs were differentiated with 8-bromo-cAMP and P4 in the presence or absence of E. PRL, IGFBP1 and 11 $\beta$ HSD1 transcript levels were determined after 4 days of treatment. The data, normalized to L19 mRNA, are expressed in arbitrary units; mean  $\pm$  SEM. \*\* indicates  $P < 0.001$





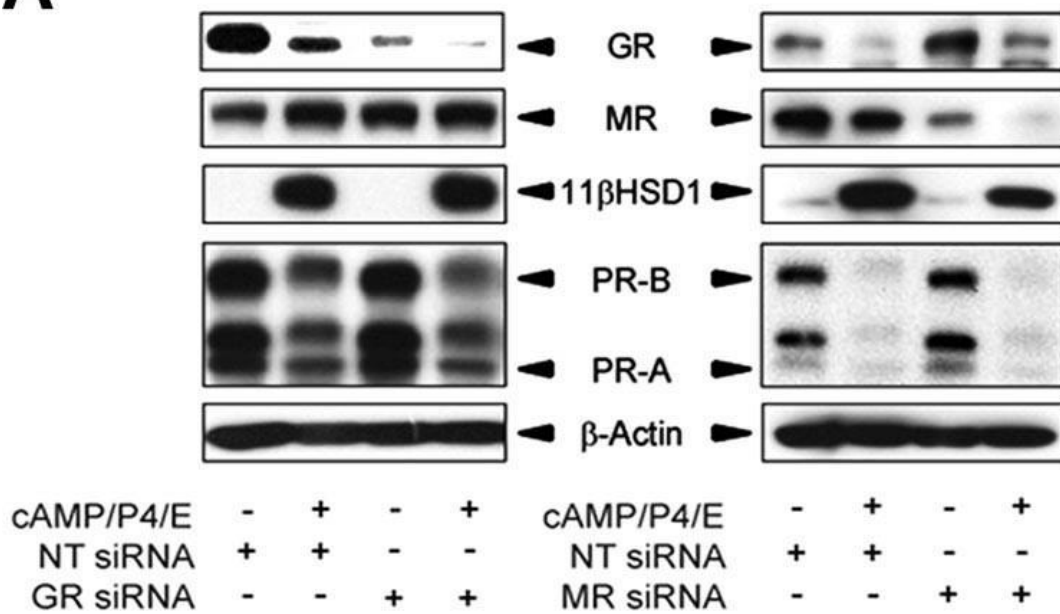
**Figure 3.5:** Effect of 11βHSD1 inhibition on the expression of decidual marker genes and GR/MR-dependent genes. Primary HESCs were differentiated with 8-bromo-cAMP, P4 and E in the presence or absence of carbenoxolone disodium salt (CBX), or PF 915275 (PF). The results show change (±SEM) in transcript levels in response to treatment with 11βHSD1 inhibitors relative to levels in undifferentiated cells (dotted lines). \*,  $P < 0.05$  and \*\*,  $P < 0.001$ .

### 3.2.3 Identification of GR and MR dependent genes in decidualizing HESCs

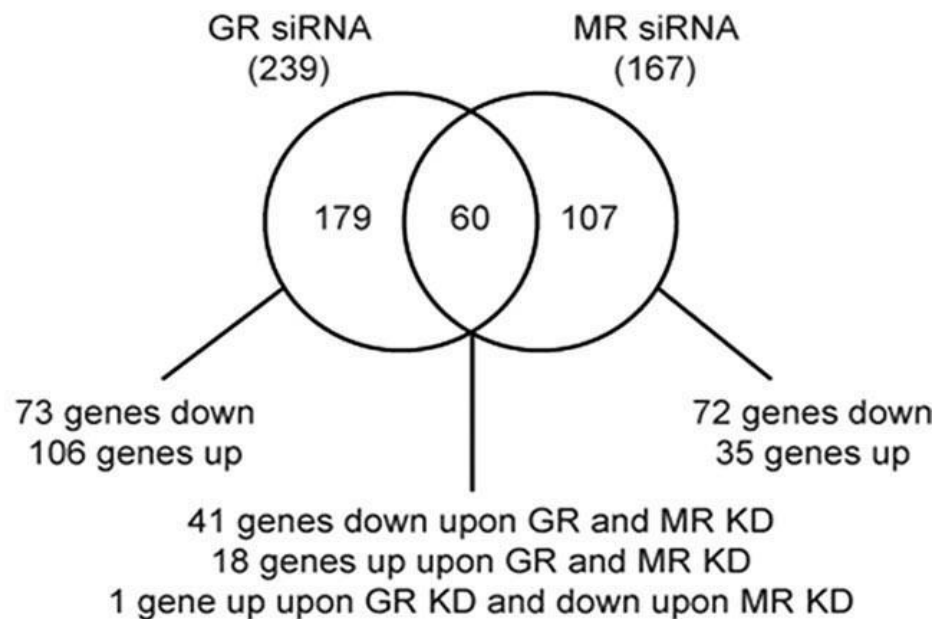
Next step was to identify GR and MR dependent genes responsive to endogenous F biosynthesis in differentiating HESCs. To do this, four individual primary cultures were first transfected with NT-, GR-, or MR-siRNA and then treated with 8-bromo-cAMP, P4, and E for 4d. Total mRNA and protein lysates were extracted from parallel cultures. Western blot analysis was used to confirm GR and MR knockdown (Fig. 3.6A). GR knockdown had no impact on MR, 11 $\beta$ HSD1, or PR levels. In contrast, MR silencing seemed to hinder the induction of 11 $\beta$ HSD1 and modestly up-regulated GR levels. Total RNA extracted from decidualizing cultures transfected with NT-, GR-, or MR-siRNA was processed for whole genome microarray analysis. Using a cut-off of more than or equal to 1.5-fold change, 179 and 107 GR and MR-selective genes, respectively, were identified ( $P < 0.05$ ) (Fig. 3.6B). Interestingly, GR knockdown resulted in significantly more up- than down-regulated genes (106 vs. 73, respectively;  $\chi^2$  test,  $P < 0.001$ ), whereas MR knockdown had the opposite effect (up- and down-regulated genes: 35 vs. 72, respectively;  $\chi^2$  test,  $P < 0.001$ ). Appendix IV, Tables 1–4 list the genes induced or repressed by GR and MR. We also identified 60 genes under control of both nuclear receptors in decidualizing HESCs with only a single gene, *GRIA1*, regulated in an opposing manner (Appendix IV, Table 5). We chose *GRIA1* as well as *WNT4* and *FBXO32* (putative GR- and MR-dependent genes, respectively) for initial validation of

the array findings. Using independent cultures, we first monitored the expression of these genes in response to treatment with 8-bromo-cAMP, P4, and E for 4 or 8 d and then examined the impact of either GR or MR knockdown. *GRIA1*, which encodes for one of the four ionotropic 2-amino-3-(3-hydroxyl-5-methyl-isoxazol-4-yl) propanoic acid receptor subunits {Sugimoto, 2010, Ionotropic glutamate receptor AMPA 1 is associated with ovulation rate}, is profoundly repressed in differentiating cells with transcript levels declining more than 90% after 4d of treatment (Fig. 3.7A). This repression was partially relieved upon GR knockdown but enhanced in response to MR silencing (Fig. 3.7B). *WNT4* and *FBXO32* are both induced upon decidualization predominantly in a GR and MR-dependent manner, respectively (Fig. 3.7). Interestingly, *FBXO32*, which encodes the E3 ubiquitin ligase Atrogin-1, was recently shown to be an 11 $\beta$ HSD1-dependent gene in skeletal muscle cells {Biedasek, 2011, Skeletal muscle 11 $\beta$ -HSD1 controls glucocorticoid-induced proteolysis and expression of E3 ubiquitin ligases atrogin-1 and MuRF-1}. In agreement, treatment of primary cultures with either CBX or PF inhibited *FBXO32* and *GRIA1* expression in decidualizing cells *PRL* (Fig. 3.5). In case of *GRIA1*, this suggests that MR-dependent induction may be dominant over GR-dependent inhibition. However, 11 $\beta$ HSD1 inhibition had no significant effect on the induction of *WNT4* in decidualizing cells. Thus, like other steroid hormone receptors, GR and MR modify decidual gene expression in a ligand-dependent and ligand-independent manner.

**A**

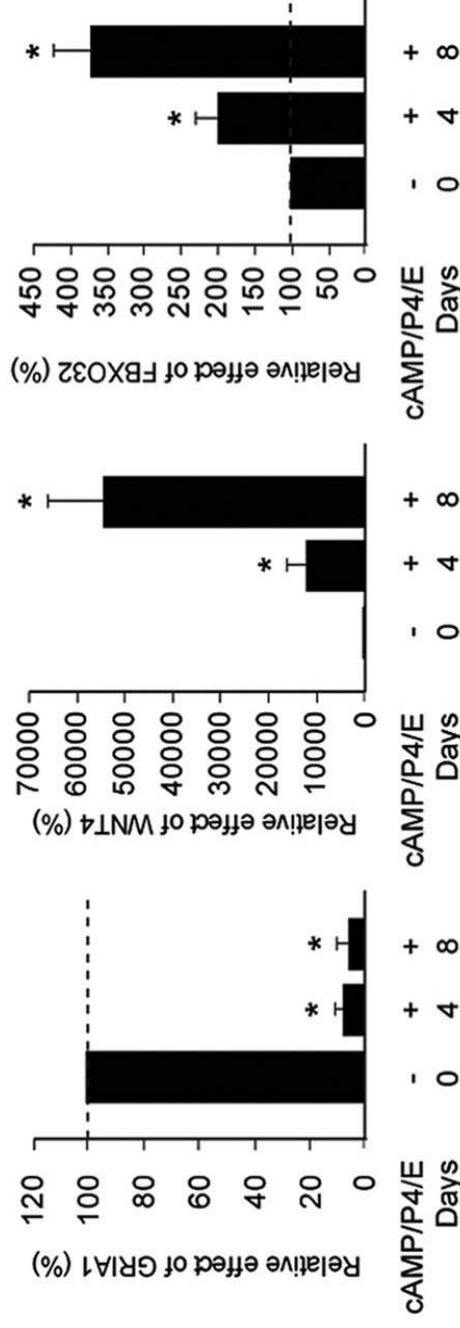


**B**

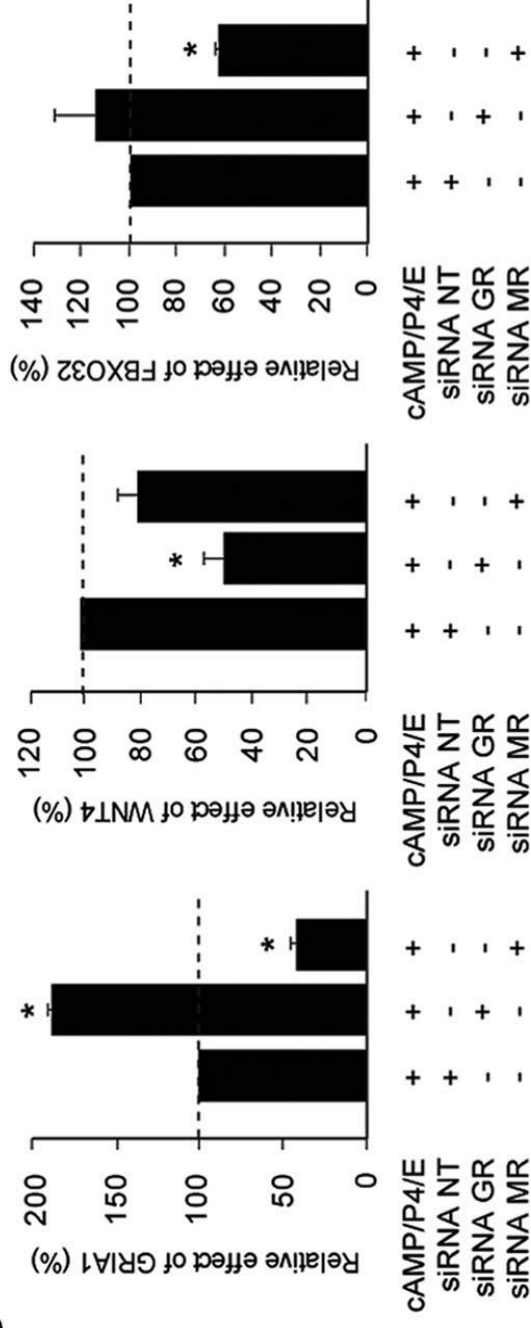


**Figure 3.6:** Identification of GR- and MR-regulated genes in decidua cells. A, Western blot analysis of GR, MR, 11βHSD1, and PR expression in protein lysates extracted from primary cultures first transfected with NT-, GR-, or MR-siRNA and then treated with 8-bromo-cAMP, P4, and E for 4 d. β-Actin served as a loading control. C, Venn diagram showing the number of differentially expressed genes in decidua cells treated with 8-bromo-cAMP, P4, and E for 4 d in response to GR or MR knockdown.

**A**



**B**



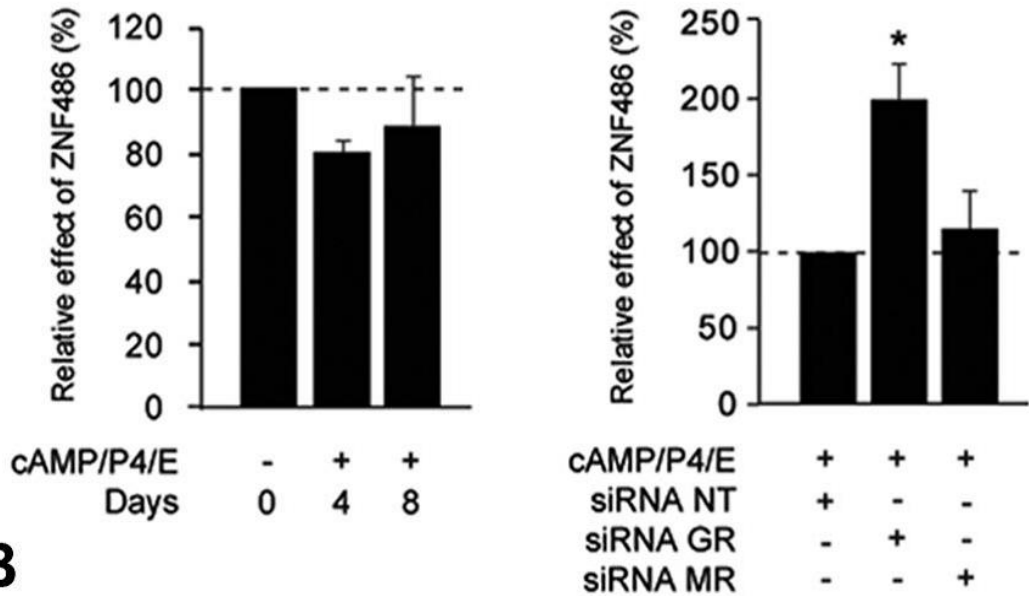
**Figure 3.7:** Expression and validation of putative GR- and MR-dependent genes in decidualizing HESCs. A, Expression of the putative GR- and MR-dependent genes in primary cultures treated 8-bromo-cAMP, P4, and E for the indicated time points. The results show mean fold change ( $\pm$  SEM) relative to levels in undifferentiated cells (dotted lines). B, Independent primary cultures (n = 3) not used in the array analysis were first transfected with NT-, GR-, or MRsiRNA and subsequently treated with 8-bromo-cAMP, P4, and E for 4 d. Transcript levels were measured by RTQ-PCR and expressed as fold-induction ( $\pm$ SEM) relative to expression levels in transfected HESCs with NT-siRNA before differentiation (dotted lines). \*  $P < 0.05$ .

### **3.2.4 GR limits the expression Krüppel-associated box domain containing ZNF (KRAB-ZNF) transcriptional repressors**

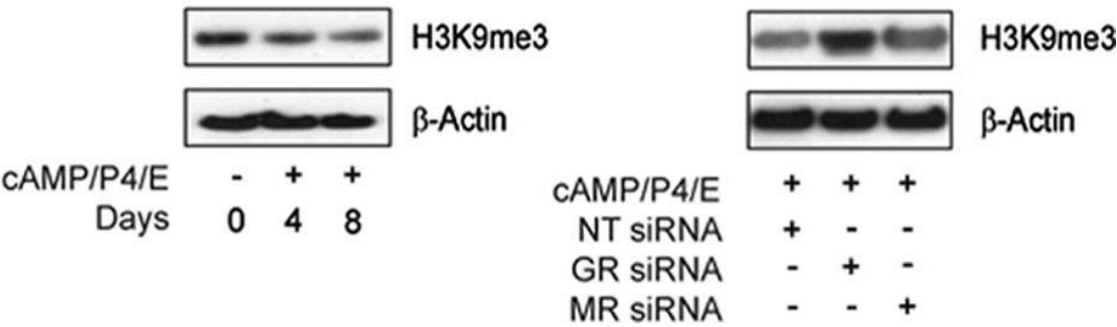
After correction for multiple testing (Benjamini and Hochberg false discovery rate), gene ontology annotation revealed that the genes repressed by GR were strongly enriched for KRAB-ZNF proteins ( $P < 0.0001$ ). In fact, 18 members of this family of transcriptional repressors were upregulated upon GR knockdown: ZNF91, ZNF92, ZNF100, ZNF253, ZNF254, ZNF311, ZNF420, ZNF484, ZNF486, ZNF585A, ZNF586, ZNF613, ZNF624, ZNF625, ZNF626, ZNF669, ZNF738, and ZNF791. Notably, the expression of five (ZNF181, ZNF223, ZNF254, ZNF625, and ZNF669) also increased upon MR knockdown (Appendix IV Tables 4 and 5). Validation analyses demonstrated that the expression of *ZNF486* transcripts declines modestly upon treatment of HESCs with 8-bromo-cAMP, P4 and E (Fig. 3.8A, *left panel*). In agreement with the array findings, GR knockdown selective up-regulated *ZNF486* mRNA levels in differentiating HESCs by approximately 2-fold (Fig. 3.8A, *right panel*). KRAB-ZNF proteins block transcriptional initiation by recruiting a variety of chromatin modifiers to promoters of target genes, resulting in an increase in H3K9me3 {Groner, 2010, KRAB-zinc finger proteins and KAP1 can mediate long-range transcriptional repression through heterochromatin spreading;Fietze, 2010, ZNF274 recruits the histone methyltransferase SETDB1 to the 3' ends of ZNF genes}. To monitor this repressive chromatin mark to determine whether GR plays a role in chromatin remodelling that underpins the decidual phenotype {Grimaldi, 2011, Down-regulation of the histone methyltransferase EZH2 contributes to the epigenetic programming

of decidualizing human endometrial stromal cells}; western blot analysis revealed a gradual decline in global H3K9me3 levels in response to differentiation cues (Fig. 3.8B, *left panel*). Knockdown of GR, and perhaps to a lesser extent of MR, was sufficient to reverse this response (Fig. 3.8B, *right panel*).

**A**



**B**



**Figure 3.8:** GR signalling plays a role in chromatin remodelling in decidualizing HESCs. A, ZNF486 transcript levels were measured in four primary cultures treated 8-bromo-cAMP, P4, and E for the indicated time points (left panel) as well as in cultures decidualized for 4 d after GR or MR knockdown (right panel). The results show mean fold change ( $\pm$ SEM)

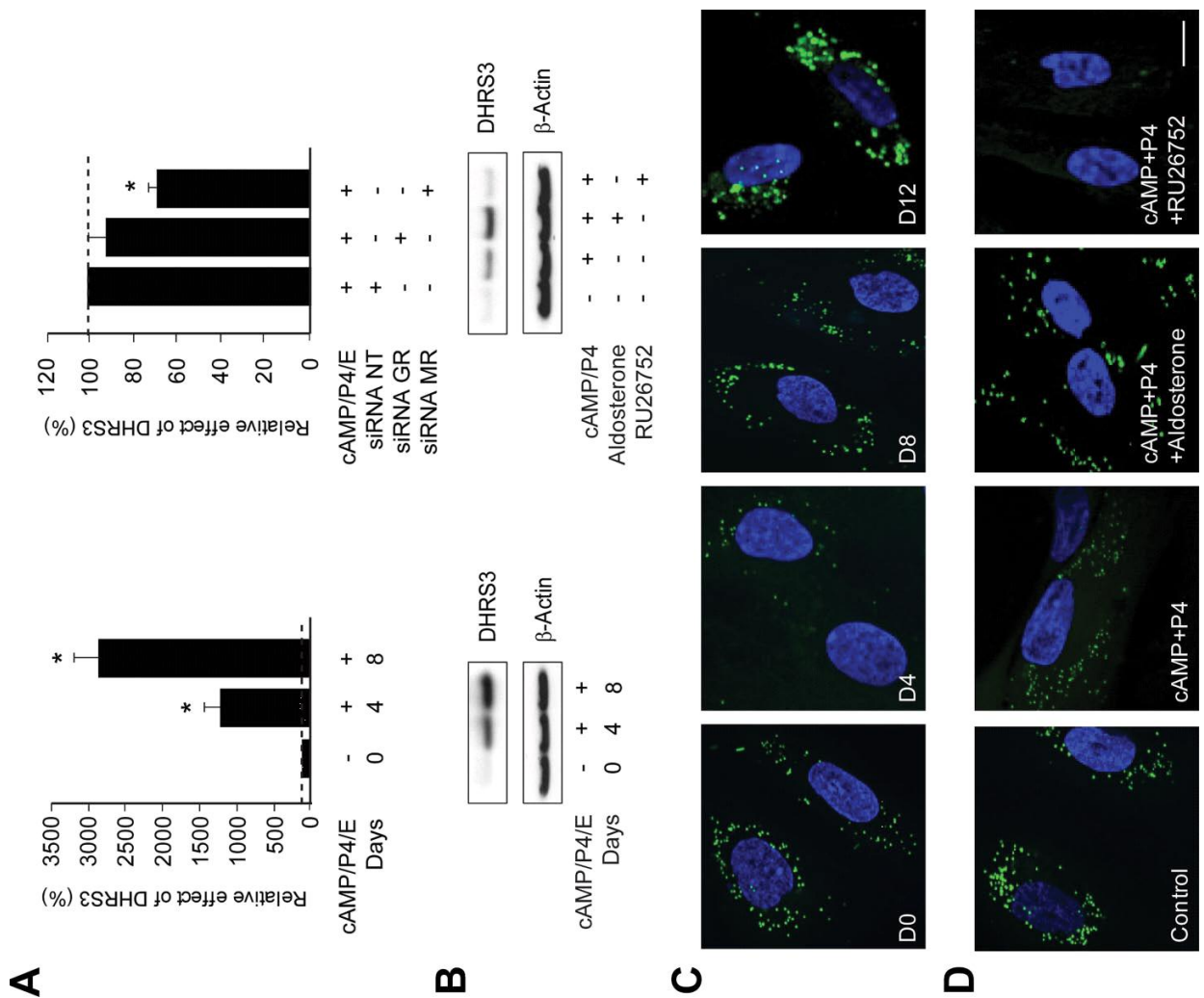
relative to levels in undifferentiated cells (dotted lines). \*  $P < 0.05$ . B, Western blot analysis of H3K9me3 expression in total protein lysates from primary cultures treated 8-bromo-cAMP, P4, and E for the indicated time points (left panel) as well as from cultures decidualized for 4 d upon GR or MR knockdown (right panel).  $\beta$ -Actin served as a loading control.

### 3.2.5 MR is essential for retinoid metabolism and lipid droplet biogenesis

In contrast to GR, MR acts primarily as a transcriptional activator in decidualizing HESCs (Appendix IV Tables 1 and 5). Perhaps the most striking observation is that several MR-induced genes encode for key enzymes involved in retinoid metabolism and cholesterol homeostasis, including retinol saturase, members of the shortchain DHRS (DHRS3, DHRS4, and DHRS4L2), the dehydrocholesterol reductase DHCR7, and the steroidogenic acute regulatory protein-related lipid transfer protein domain containing protein STARD5. To validate the array findings, we focused on DHRS3 (also known as retinal short-chain dehydrogenase/reductase 1). This enzyme was highly induced at both mRNA and protein level in a time dependent manner upon treatment of primary HESCs with 8-bromo-cAMP, P4, and E (Fig. 3.9A). MR knockdown attenuated the induction of *DHRS3* transcripts in decidualizing cells (Fig. 3.9B, *right panel*). To further explore MR dependency of *DHRS3* expression in decidualizing cells, primary cultures were treated with 8-bromo-cAMP, P4, and either aldosterone or RU26752, a MR antagonist (Agarwal, 1999; Kelly, 2011). Although addition of aldosterone potentiated the induction DHRS3 in cells differentiated with 8-



bromo-cAMP and P4, RU26752 completely abolished this response (Fig.3.9B, *right panel*). DHRS3 has recently been implicated in endoplasmic reticulum (ER)-derived lipid droplet formation (Deisenroth, 2011). Loading of primary HESCs with a cell-permeable lipophilic fluorescence dye, BODIPY 493/503, followed by confocal microscopy demonstrated that Decidualization is associated with highly dynamic changes in the appearance of these cytoplasmic lipid droplets. Undifferentiated HESCs contain numerous small droplets. Unexpectedly, these lipid droplets were consistently less abundant after 4d of differentiation (Fig.3.9C). By day 8, the droplets had re-accumulated especially near the periphery of the cells. Four days later, fewer but larger and more centrally localized lipid droplets were present in decidualizing HESCs (Fig. 3.9C). Next, we treated primary cultures with 8-bromo-cAMP, P4, and either aldosterone or RU26752 for 8d. Interestingly, although addition of aldosterone favoured the formation of larger droplets, RU26752 virtually abolished their presence all together (Fig. 3.9D).



**Figure 3.9:** MR-dependent induction of DHR33 in decidualizing HESCs is associated with dynamic changes in cytoplasmic lipid droplets. DHR33 expression was examined in parallel primary cultures treated with 8-bromo-cAMP, P4, and E for the indicated time points as well as after GR or MR siRNA knockdown by RTQ-PCR (A) or Western blot analysis (B). DHR33 expression was analyzed in four independent primary cultures. The left panel shows percentage change ( $\pm$ SEM) in mRNA expression relative to levels in undifferentiated cells (dotted lines). C, Confocal micrographs showing lipid droplets stained with BODIPY (green) and nuclei stained with 4,6-diamidino-2-phenylindole (DAPI) (blue) in HESCs treated with 8-bromo-cAMP, P4, and E for the indicated time points. D, Representative confocal images of HESCs decidualized with 8-bromo-cAMP and P4 in the presence or absence of aldosterone or RU26752 for 8 d. Scale bar, 10  $\mu$ m.

### 3.3 DISCUSSION

Elevated circulating P4 levels maintain the maternal decidual response in pregnancy and are therefore indispensable for survival of the fetus. However, circulating levels of androgens, aldosterone, and F also increase in early pregnancy (Gennari-Moser, 2011; Jensen, 2002; Castracane, 1998; Cloke, 2012). Furthermore, the endometrium expresses the full complement of cognate receptors as well as a host of steroidogenic enzymes that will determine the local bioavailability of different ligands. For example, increased expression accounts for the higher conversion of testosterone to dihydrotestosterone in secretory compared with proliferative endometrium (Ito, 2002; Cloke, 2008). From the experiments, P4 drives 11 $\beta$ HSD1 expression, which is further reinforced by F production and signalling. This positive feedback mechanism renders 11  $\beta$ HSD1 one of the most highly induced genes upon HESC differentiation (Takano, 2007). Further, decidualization is also associated with a rebalancing of corticosteroid receptors in favour of MR. MR and GR show significant amino acid homology in their ligand- and DNA-binding domains with PR and androgen receptor (AR) (Funder, 1997). Together, these four receptors constitute the 3-ketosteroid receptor subfamily of nuclear receptors. The structural homology between these receptors implies that a degree of promiscuity may exist in the binding of various natural or synthetic ligands. This is indeed the case. For example, P4 binds MR, although the physiological consequences of this interaction, if any, are still unknown (Myles, 1996). Similarly, MPA, which unlike P4 does not have the propensity to partition onto glass and plastic in culture, is widely used to study PR responses *in vitro*, yet it is also a potent

activator of GR and AR (Selman, 1996; Cloke, 2008). Structural homology also raises the possibility of functional redundancy between these receptors. Previous study has shown that this is not the case for PR and AR (Ito, 2002). By combining siRNA-mediated knockdown with genome-wide expression profiling, it is clear that AR signalling is essential for cytoskeletal organization and cell cycle regulation in decidualizing cells. PR knockdown deregulated approximately nine times more genes than AR silencing (92 vs. 860 genes, respectively). Interestingly, a significant number of PR-regulated genes encode for membrane bound receptors and intermediates in various signal transduction pathways, suggesting that P4 dependency of the decidual phenotype is, at least in part, accounted for by PR-dependent reprogramming of pathways activated by growth factors and cytokines (Cloke, 2008). A similar approach was adopted to identify MR- and GR-dependent genes in differentiating HESCs. Instead of using MPA, as it introduces a bias in favour of GR responses, P4 in combination with 8-bromo-cAMP was used and added E as the substrate for 11 $\beta$ HSD1 conversion. After 4d of treatment, we identified 179 and 107 deregulated genes upon GR or MR knockdown, respectively. Like PR (Cloke, 2008), GR represses significantly more genes than it induces in decidualizing cells. The number of up- and down-regulated genes upon AR knockdown is comparable, whereas MR functions primarily to promote the expression of certain decidual genes. Cross-referencing of the array data identified only 12 genes that are regulated by PR or AR as well as GR or MR (Appendix IV, Table 6). Mining of the GR-dependent genes yielded some unexpected results. For example, GR represses the expression of *SPP1* (osteopontin), a

major component of the embryo-endometrial interaction (Loke, 1995), suggesting that glucocorticoid exposure during the window of implantation may interfere with embryo implantation. GR stimulated the induction of WNT4, a key component of P4 responses in both the uterus and breast (Briskin, 2000; Franco, 2011). Further, GR as well as MR signalling may be important for sustained cAMP activity in decidualizing cells by up-regulating the  $\alpha$ -catalytic subunit of protein kinase A (*PRKACA*) (Appendix IV, Table 5). However, the most striking observation was that GR activity attenuates the induction of 18 KRAB-ZNF proteins. With 675 encoding genes, the C2H2 zinc-finger proteins comprise the largest family of regulatory proteins in mammals, and 36% contain a KRAB domain (Fietze, 2010; Rousseau-Merck, 2002; Vaquerizas, 2009). Over 50% of all human KRAB-ZFP genes are located in clusters on chromosome 19, including 15 of the 18 genes found to be regulated by GR in this study. The functions of these GR-repressed KRAB-ZFP genes are unknown with the exception of *ZNF420*, which encodes ATM and p53-associated krüppel type zinc finger protein, a negative regulator of p53-mediated apoptosis (Tian, 2009). However, it is well established that the KRAB domain confers a potent transcriptional repressor function by mediating specific interactions with a co-repressor protein, krüppel -associated protein 1 (encoded by *TRIM28*), which in turn serves to recruit chromatin deacetylation machinery (Moosmann, 1996; Schultz, 2002; Ayyanathan, 2003), as well as methyltransferase complexes (Fietze, 2010). Because KRAB-ZNF transcription factors have been implicated in trimethylating H3K9 (Fietze, 2010), the cellular levels of this histone mark upon differentiation of HESCs was monitored. Perhaps

somewhat fortuitously, this line of inquiry showed that decidualization is associated with a decline in global cellular H3K9me3 levels, which is disrupted upon GR knockdown. Analysis of genes deregulated upon MR knockdown highlighted the dynamic changes in lipid droplet formation and retinoid metabolism that occur upon decidual transformation of HESCs. Although the MR dependency of these metabolic functions in decidualizing HESCs was unanticipated, it is in keeping with the observation that silencing of this nuclear receptor in murine adipocytes completely prevents lipid accumulation (Hoppmann *et al.*, 2010). In contrast, GR knockout only mildly impairs adipogenesis. Aldosterone has been shown to promote adipose conversion of 3T3-L1 and 3T3-F442A cells, whereas DEX inhibits terminal adipocyte maturation (Caprio, 2007). Further, two MR-dependent genes in decidualizing HESCs, *RETSAT* and *DHRS3*, are strongly implicated in both intracellular lipid accumulation and retinoid metabolism (Deisenroth, 2011; Schupp, 2009).

Retinoic acid (RA), the biologically active metabolite of vitamin A (retinol), is essential for embryogenesis and maintenance of pregnancy. Both RA deficiency and excess causes severe fetal malformation, suggesting that retinoid metabolism must be tightly controlled at the fetomaternal interface (Niederreither, 2008; Xia, 2010; Han, 2010). RA is derived from oxidation of all-*trans*-retinaldehyde (retinal), an unstable intermediate that fluxes between retinol and RA. Because *DHRS3* is a retinaldehyde reductase that promotes storage of retinol in lipid droplets, its level of expression may be an important mechanism to modulate local RA availability (Cerignoli, 2002; Deisenroth,

2011). DHRS3 levels are low in undifferentiated HESCs, but expression increases markedly upon decidualization, in parallel with the induction of 11 $\beta$ HSD1. In fact, both enzymes are structurally related members of the SDR superfamily that localize to the ER (Maser, 1997). The terminal enzymes involved in the synthesis of lipid droplets also localize to the ER and often to droplets themselves, as is the case for DHRS3 (Deisenroth, 2011). Thus, the dynamic changes in the appearance and abundance of lipid droplets in decidualizing HESCs may at least partly reflect the changing nature of ER-resident enzymes. Lipid droplets are often viewed as mere energy storage facilities, containing predominantly neutral lipids and various proteins (Goodman, 2008). Based on our observations, it is tempting to speculate that the constituents and functions of these lipid droplets also change upon decidualization of HESCs, perhaps becoming more akin to the retinyl ester storage particles (retinosomes) present in the eye (Orban, 2011). In summary, the observations suggest that decidualization of the endometrium promotes the formation of a corticosteroid gradient at the feto-maternal interface. Although F is virtually absent in the placenta due to the abundant expression of 11 $\beta$ HSD2 (Shams, 1998; McCalla, 1998), its production on the maternal side may exert important autocrine as well as paracrine functions. For example, local F biosynthesis could constitute a major mechanism that protects the fetal allograft against a potential maternal immune response. Importantly, the data also suggest that MR is a central regulator of the metabolic functions of the maternal decidua. This may be of particular importance to human pregnancy, because perfusion of the placenta is not established until 10–12 weeks of gestation (Jauniaux, 2000).

Consequently, throughout the process of organogenesis (3–8 weeks of pregnancy), fetal nutrition depends entirely on secretions produced by endometrial glands and decidualizing stroma (Burton, 2011; Spiegler, 2012). The role of the endometrial 11 $\beta$ HSD1/GR/MR pathway in reproductive failure clearly warrants further investigation.



**Chapter 4: Elevated  
peri-implantation uterine natural  
killer cell density in human  
endometrium is associated with  
impaired decidualization stromal  
cells in recurrent miscarriage and  
recurrent implantation failure  
patients**

## 4.1 INTRODUCTION

Successful implantation is a subtle dialogue between the maternal endometrium and the embryo. A receptive endometrium is a prerequisite from the maternal side (Giudice, 1999; Navot, 1991) which lasts for a limited period of time. The luminal epithelium undergoes precisely defined morphological changes until a receptive endometrium is developed. The 'implantation window' is described as the period in the mid-luteal phase from day 19 to day 24, when implantation can take place (Navot, 1991; Dominguez, 2003) and is time limited (Wilcox, 1999). This corresponds to the time when the embryo hatches, 6 days after LH surge, and consequently ready for implantation within the following 24 hours . Although the luminal endometrial epithelium is the primary barrier in the implantation process, the progesterone responses in this cellular compartment that underpin the receptive phenotype are mediated by signals derived from the underlying stromal cells (Simon, 2009), i.e. from decidualization of the stromal compartment.

Decidualization is a progressive process occurring in the late mid secretory phase in a temporal and spatial fashion comprising of morphologic, biochemical, and vascular modifications initiated by the presence of progesterone after oestrogen priming (Ramathal, 2010). The implanting blastocyst initially apposes and attaches to the luminal epithelium of the endometrial lining about 6–7 days after conception. Soon after the epithelium is breached, the interface lies between trophoblast and decidual cells. The trophoblast invasion extends beyond the endometrium into the uterine

junctional zone, which is the inner third of the myometrium (Brosens, 2010; Brosens, 1995). The decidua–trophoblast dialogue orchestrates the remarkably dynamic process by which the blastocyst becomes completely embedded in the uterine wall within a few days, as early as 10 days after LH surge (Norwitz, 2001; de Ziegler, 1998). A defect predisposes to related pregnancy complications, including miscarriage, preeclampsia, fetal growth restriction, and preterm labour. Inadequate uterine receptivity is thought to be responsible for two-thirds of implantation failures (Simón, 1998).

Uterine natural killer (uNK) cells, an important component of the innate immune system, are the most abundant immune cells in the mid-luteal (peri-implantation) endometrium and in the decidua of early pregnancy (Manaster, 2008). They represent a unique subset of NK cells; staining brightly for CD56 (CD56+) but dimly for CD16 (CD16-). uNK cells play a significant role in the establishment and maintenance of early pregnancy by promoting decidual angiogenesis, spiral arterial remodeling and trophoblast invasion (Hanna, 2006; Quenby, 2009). In contrast to their circulating (CD56+/CD16+) counterparts, there is little evidence for a cytotoxic role of uNK cells at the feto-maternal interface. However, uNK express killer-cell immunoglobulin-like receptors (KIR) that preferentially bind to human leukocyte antigen (HLA)-C molecules expressed on placental cells, suggesting a role in maternal allorecognition of fetal trophoblast. They are abundant around the spiral arteries, near endometrial glands and adjacent to extravillous trophoblast in early pregnancy. Thus, uNK cells are unique in terms of their tissue distribution, phenotype and function. Also, resident human endometrial stromal cells (HESCs) are thought to serve as gatekeepers for the

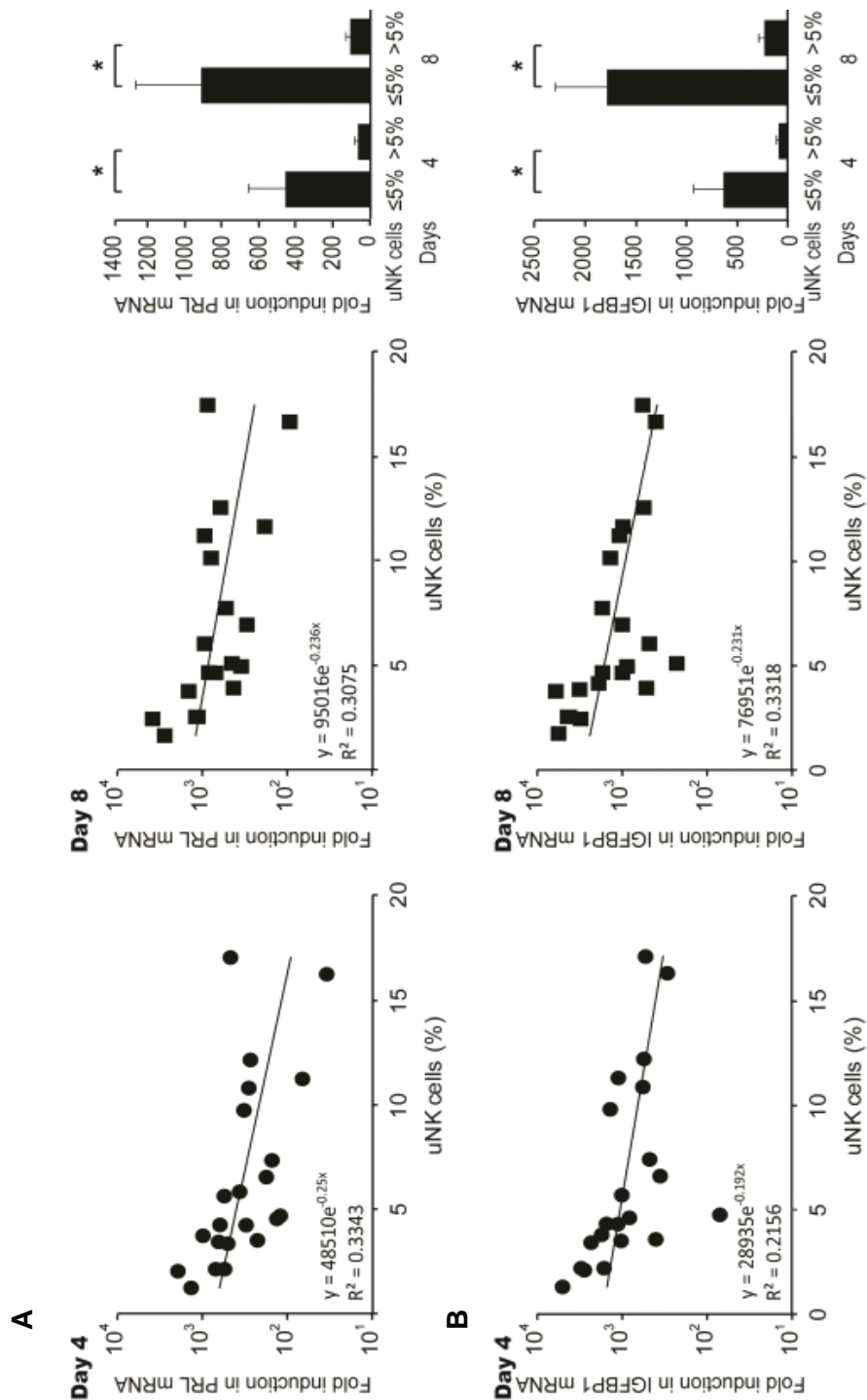
recruitment and distribution of immune cells in the peri-implantation endometrium . For example, decidualizing (differentiating) HESCs secrete interleukin 11 (IL-11) and IL-15, two multifaceted cytokines implicated in trafficking and differentiation of uNK cells.

Both the maternal KIR and fetal HLA-C gene systems are highly polymorphic and certain genotypic combinations are associated with a modest increase or decrease in pregnancy complications, including miscarriage, fetal growth restriction and pre-eclampsia. In addition, several studies reported an association between elevated uNK cell levels in mid-luteal endometrium and reproductive failure (Chazara, 2011; Quenby, 2009; Clifford, 1999). In particular, there is compelling evidence to link increased uNK density to recurrent pregnancy loss (RPL), defined here as three or more consecutive miscarriages. RPL is a prevalent disorder that affects 1–2% of couples and a cause of considerable physical and psychological morbidity (Rai, 2006). Furthermore, RPL is associated with increased likelihood of obstetric complications and adverse perinatal outcome in a subsequent on going pregnancy . Whether mid-luteal uNK cell testing in a non-conception cycle predicts subsequent pregnancy complications remains unresolved .

## 4.2 RESULTS

### 4.2.1 Elevated uNK cell density *in vivo* is associated with impaired induction of key decidual markers *in vitro*

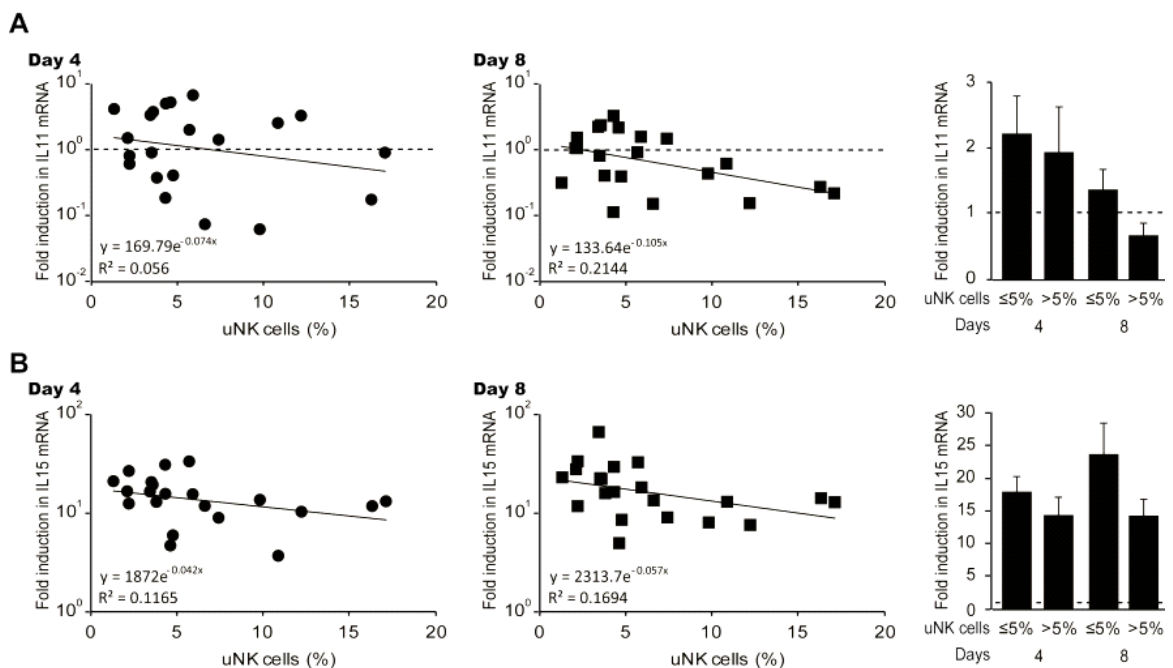
Previous studies have shown that an aberrant decidual response, associated with reproductive disorders such as endometriosis and recurrent pregnancy loss (RPL), is maintained in culture (Klemmt, 2006; Salker, 2012). This prompted further investigation into the possibility of uNK cell density *in vivo* reflecting the induction of 11 $\beta$ HSD1 in response to decidual cues. For this, timed endometrial biopsies were divided and processed as follows: one part for CD56 immunostaining and the other for primary HESCs. These cultures were passaged once, grown to confluency, and treated with 8-bromo-cAMP, P4 and E for either 4 or 8 days. As shown in Figure 4.1, there was a striking inverse correlation between uNK cell density *in vivo* and the responsiveness of paired primary cultures to differentiation stimuli. This inverse correlation was noted for *PRL* and *IGFBP1*, two 'classical' decidual marker genes (Fig. 4.1A & 4.1B).



**Figure 4.1:** Inverse correlation between uNK cell densities in vivo and the induction of decidua markers in vitro. A, The uNK cell densities in midluteal biopsies correlated inversely to the induction of PRL transcripts in primary HESCs decidualized for 4 days (left panel) or 8 days (middle panel). The right panel shows the mean (SEM) induction of PRL transcripts in biopsies deemed to have normal or elevated uNK cell counts. Note the logarithmic Y-axis. B, The uNK cell densities in midluteal biopsies correlated inversely to the induction of IGFBP1 transcripts in primary HESCs decidualized for 4 days (left panel) or 8 days (middle panel). The right panel shows the mean (SEM) induction of PRL transcripts in biopsies deemed to have normal or elevated uNK cell counts. Note the logarithmic Y-axis. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

## 4.2.1 Cytokines IL-11 and IL-15

Upon examining whether this association extended to IL-11 and IL-15, cytokines implicated in regulating uNK cells (Ain, 2004; Ashkar, 2003; Ain, 2004; Ashkar, 2003; Godbole, 2010). Rather surprisingly, there was a trend towards higher levels of induction of IL-11 and IL-15 transcripts in decidualizing HESC cultures obtained from biopsies with normal uNK cell densities (Fig. 4.2A & 4.3B), although this did not reach statistical significance ( $P > 0.05$ ).



**Figure 4.2:** Induction of IL11 and IL15 mRNA in decidualizing HESCs in culture does not correlate to uNK cell densities in vivo. A & B, the uNK cell densities in 21 biopsies were correlated to the induction of IL11 and IL15 transcripts, respectively, in corresponding primary HESCs decidualized either for 4 days (left panel) or 8 days (middle panel). The right panel shows the mean ( $\pm$  SEM) induction in biopsies deemed to have normal or elevated uNK cell counts.

## 4.3 DISCUSSION

Increased uNK cell density in mid-luteal endometrium has been associated with reproductive failure, especially RPL (Chazara, 2011; Quenby, 2009; Clifford, 1999). However, the mechanisms that account for cyclic recruitment of uNK cell precursors and subsequent proliferation and differentiation within the peri-implantation environment are not well understood. In addition to IL-11 and IL-15, several other endometrial factors may be implicated in this process, including chemokine (C-X-C) motif ligand 14, IL-12 and IL-33 (Salker, 2012; Karsten, 2009; Mokhtar, 2010). Notably, the decidual process in the human endometrium is under tight spatiotemporal control (Gellersen, 2007). It is initiated in the mid-luteal phase of the cycle first in stromal cells surrounding the terminal spiral arteries and underlying the luminal epithelium. Thus, rather than the total number of uNK cells, it is possible that excessive migration of uNK cells from their usual position in the basal and peri-vascular regions of the endometrium to the sub-luminal region is the hallmark of an abnormal decidual response that predisposes for early pregnancy loss. Finally, there is increasing evidence that the responsiveness of endometrial cells to differentiation signals is subjected to epigenetic programming (Grimaldi, 2011), which explains how an aberrant decidual response *in vivo* is maintained, at least partly, upon differentiation of purified HESCs *in vitro* (Klemmt, 2006; Salker, 2012; Aghajanova, 2010; Salker, 2011). In agreement, high uNK cell density *in vivo* is associated with blunted induction decidual marker genes, such as *PRL* and *IGFBP1*, in primary cultures. Somewhat paradoxically, induction of IL-11 and IL-15 also tended to be lower in decidualizing cells established from biopsies with elevated uNK cell



levels, although this trend was not significant. This observation does not exclude the possibility that expression levels of these cytokines *in situ* correlate with uNK cell levels as reported for IL-15 in a recent study .

Human uNK cells have been described as immature and inactive before pregnancy (Manaster, 2008). It is unclear as to how high uNK cell densities prior to conception predisposes to subsequent pregnancy failure. Ablation of these cells in mice has been shown to compromise spiral arteriole remodelling and maintenance of decidual integrity seen after mid-pregnancy. Yet uNK cell deficient  $IL15^{-/-}$  mice are fertile, have normal gestation lengths and litter sizes comparable to wild-type mice . Similarly, human uNK cells are implicated in spiral artery remodelling. They are a rich source of angiogenic growth factors, although paradoxically the endometrium of RPL patients is characterized by reduced expression of several key factors, including PDGF-BB, Ang-2, VEGF-A and VEGF-C .

It seems likely that complex and dynamic gradients of chemoattractants and chemorepellents control the spatiotemporal distribution of uNK cells in the peri-implantation endometrium. The above data suggest that excessive uNK cells in the subluminal stroma compartment prior to conception may serve as a potential biomarker for a suboptimal decidual response in pregnancy.

**Chapter 5: Elevated  
peri-implantation uterine natural  
killer cell density in human  
endometrium is associated with  
impaired corticosteroid  
signalling in decidualizing  
stromal cells**

## 5.1 INTRODUCTION

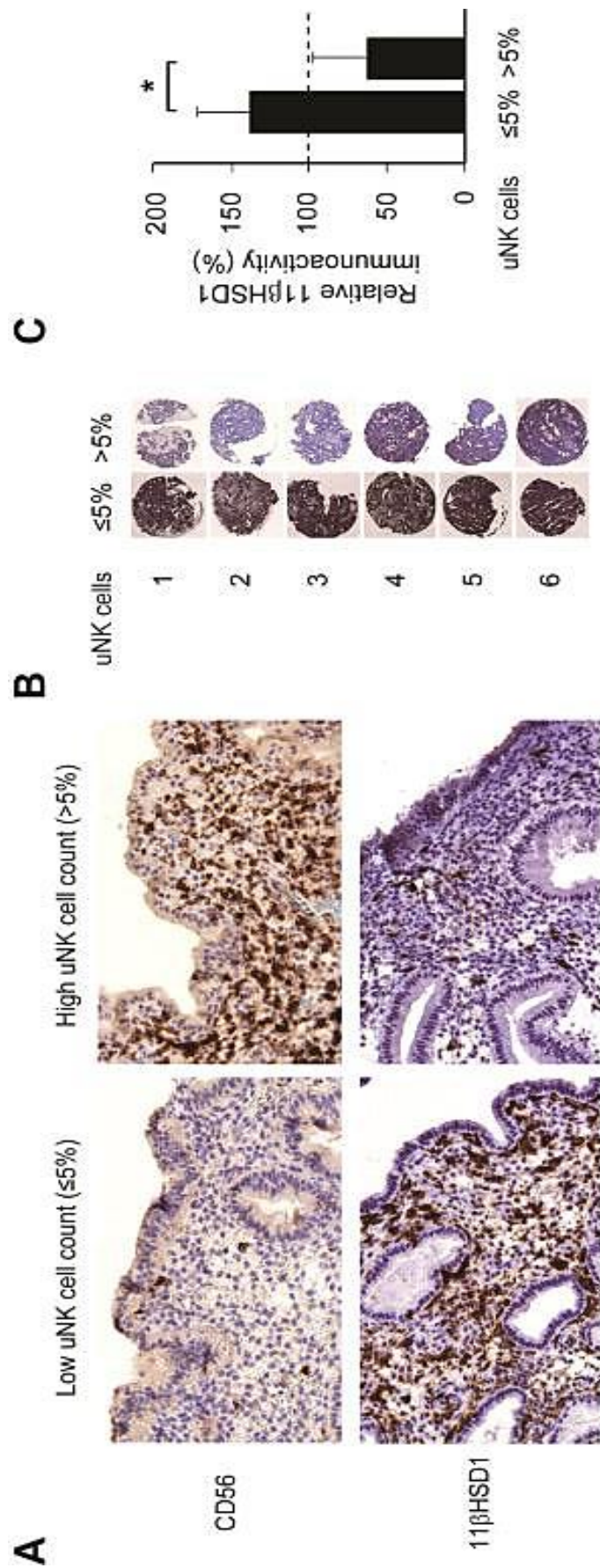
Uterine NK (uNK) cells express the glucocorticoid receptor (GR) but lack progesterone receptor (PR), rendering them directly responsive to cortisol but not progesterone (Henderson, 2003; Guo, 2012). Consistent with this notion, pre-conceptual glucocorticoid (prednisolone) treatment significantly reduces uNK cell density in RPL subjects as well as endometrial angiogenesis . Progesterone massively enhances the expression and activity of 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1) in decidualizing HESCs as shown in Chapter 3, , suggesting that local cortisol biosynthesis plays an integral role in the preparation of the endometrium for implantation. Decidualization is further associated with a decline in GR expression and reciprocal induction of the mineralocorticoid receptor (MR), which in turn drives the expression of several key enzymes involved in lipid and retinoid metabolism, including retinol saturase (RETSAT), various short-chain dehydrogenases/reductases (SDRs; such as DHRS3, DHRS4 and DHRS4L2), and StAR-related lipid transfer protein 5 .

Emerging evidence suggests that aberrant differentiation of resident HESCs into specialist decidual cells is the hallmark of RPL. Taken together, these observations raises the possibility that excessive uNK cell levels in mid-luteal endometrial samples may reflect relative local corticosteroid deficiency, caused by inadequate induction of decidual 11 $\beta$ HSD1 and resulting in impaired local metabolic function.

## **5.2 RESULTS**

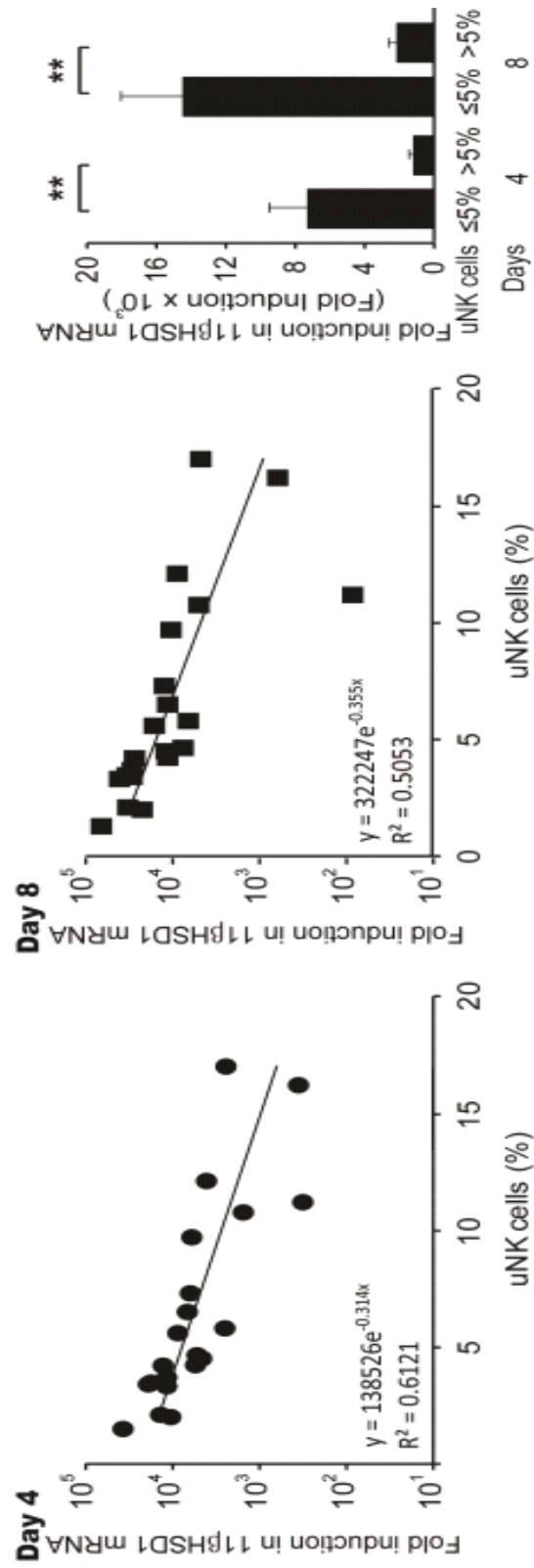
### **5.2.1 Mid-luteal uNK cell density correlates inversely with endometrial 11 $\beta$ HSD1 expression**

Routinely uNK cell densities are assessed by CD56 immunostaining of timed (days LH+7 to LH+10) endometrial biopsies from women suffering reproductive failure. Based on previous studies, normal uNK cell density is defined as 5% CD56+ cells in the stroma underlying the luminal epithelium. Hence, apparent excess of uNK cells in the peri-implantation endometrium may reflect impaired 11 $\beta$ HSD1 expression and relative cortisol deficiency. To test this hypothesis, a TMA was constructed using biopsies with normal (n=18) as well as elevated (n=18) uNK cell levels. Consecutive tissue sections were stained with anti-CD56 and anti-11 $\beta$ HSD1 antibodies, respectively. As shown in Figure 5.1A, a strong inverse correlation was observed between uNK cell density and 11 $\beta$ HSD1 immunoreactivity. In fact, this negative correlation was apparent at low magnification (Fig. 5.1B) and confirmed by semi-quantitative image analysis (Fig. 5.1C).



**Figure 5.1:** Inverse correlation between uNK cell density in the subluminal epithelium and expression of 11βHSD1 is noted. A, representative CD56 and 11βHSD1 immunostaining in mid-luteal endometrial biopsies deemed to have normal or elevated uNK cell count ( $< 5\%$  or  $> 5\%$  CD56+ cells in the stroma, respectively). Original magnification X 20. B, low magnification showing the intensity of 11βHSD1 immunostaining in representative needle cores of endometrial biopsies with normal or elevated uNK cell densities. C, image analysis of 11βHSD1 immunostaining of a TMA containing biopsies with normal ( $< 5\%$ ; n=18) or increased ( $> 5\%$ ; n=18) uNK cell density in the subluminal stroma.

Previous studies have shown that an aberrant decidual response associated with reproductive disorders such as endometriosis and recurrent pregnancy loss (RPL), is maintained in culture. This prompted us to investigate if uNK cell density *in vivo* could reflect the induction of 11 $\beta$ HSD1 in response to decidual cues. To this end, we divided timed endometrial biopsies and processed one part for CD56 immunostaining and the other for primary HESCs. These cultures were passaged once, grown to confluency, and treated with 8-bromo-cAMP, P4 and E for either 4 or 8 days. As shown in Figure 5.2, there was a striking inverse correlation between uNK cell density *in vivo* and the responsiveness of paired primary cultures to differentiation stimuli. This inverse correlation was not only apparent for the induction of *HSD11B1*, the gene that encodes 11 $\beta$ HSD1 (Fig. 5.2A), but also for *PRL* and *IGFBP1*, two 'classical' decidual marker genes (Fig. 5.2B & 2C) as already seen earlier.



**Figure 5.2:** Inverse correlation between uNK cell densities in vivo and the induction of decidual markers in vitro. A, The uNK cell densities in midluteal biopsies correlated inversely to the induction of 11HSD1 transcripts in primary HESCs decidualized for 4 days (left panel) or 8 days (middle panel). Note the logarithmic Y-axis. The right panel shows the mean (SEM) induction of 11HSD1 transcripts in biopsies deemed to have normal or elevated uNK cell counts.

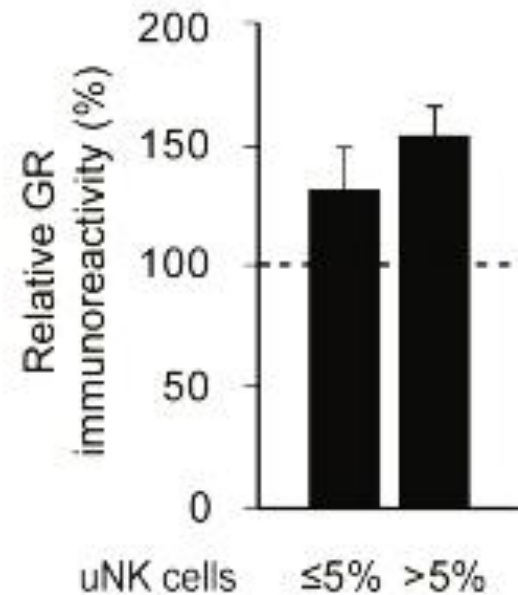
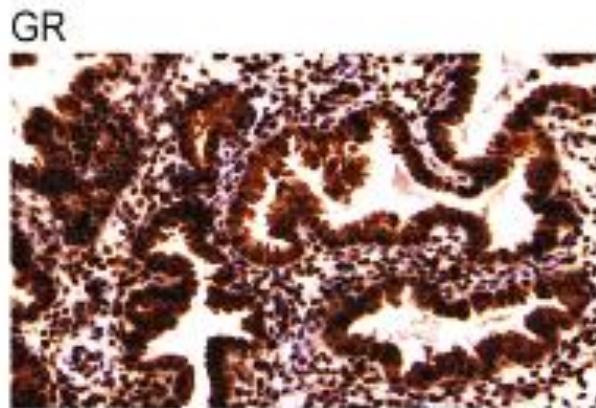
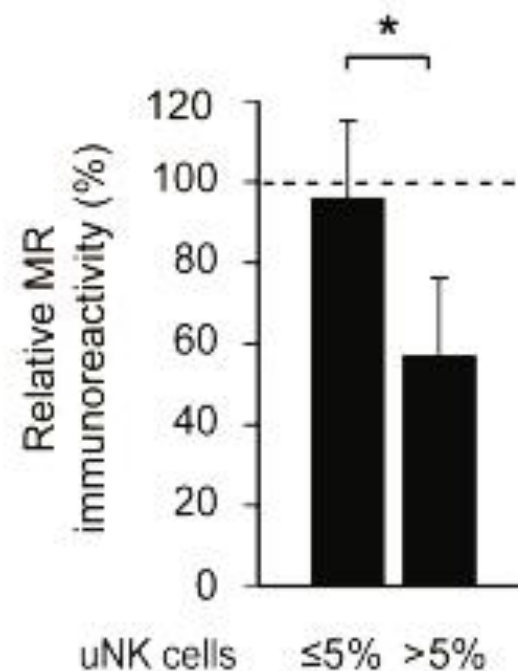
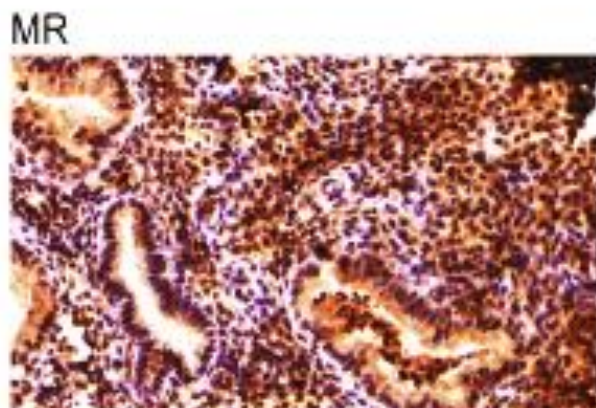
### **5.2.1 Elevated uNK cell density in vivo is associated with impaired expression of MR- dependent metabolic enzymes**

The TMA was examined for the expression of the cortisol-responsive receptors; GR and MR. As reported by others , MR and GR are both expressed in the endometrial stroma (Fig. 5.3A). Semi-quantitative analysis of the TMA showed no difference in stromal GR immunoreactivity in biopsies characterized elevated versus normal uNK cell levels. In contrast, increased % CD56+ cell density was associated with significant lower MR expression levels ( $P < 0.05$ ; Fig. 5.3B).

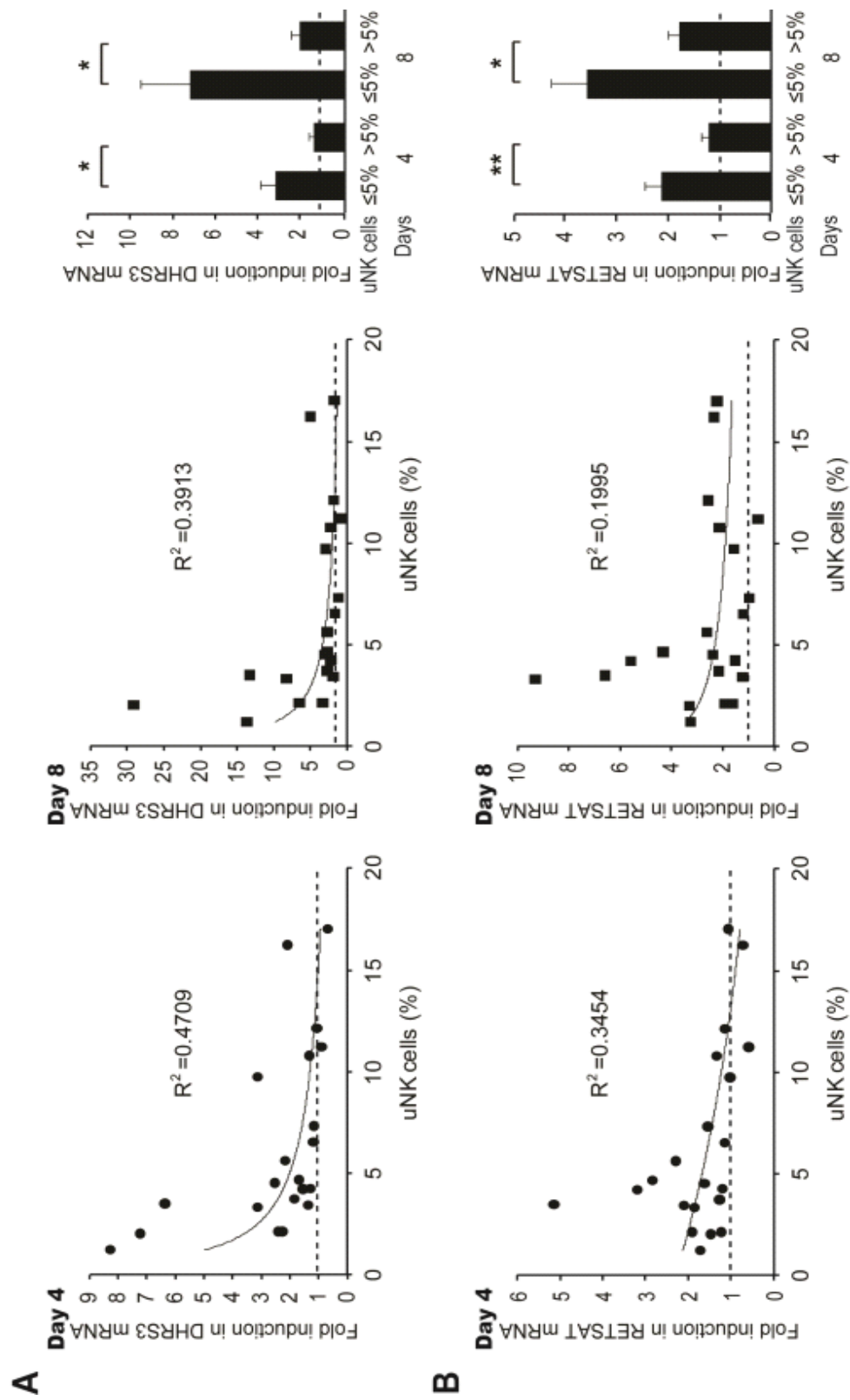
Previous knockdown experiments (as described in chapter 3) have shown that the 11 $\beta$ HSD1/MR axis in decidualizing HESCs regulates the expression of several enzymes involved in lipid metabolism and retinoid acid biosynthesis and storage (293). The expression levels of two target genes, *DHRS3* and *RETSAT*, to monitor the MR activity in decidualizing primary HESC cultures established from biopsies with normal or elevated uNK cell levels were measured. Both genes were moderately induced upon treatment with 8-bromo-cAMP/P4/E in a time-dependent manner when the primary HESCs cultures were established from samples with 5% CD56+ cells in the subepithelial stroma (Fig. 5.4). By contrast, this induction was negligible or absent in cultures established from high uNK cell samples. To validate these findings, we performed Western blot analysis of primary HESCs decidualized with 8-bromo-cAMP/P4/E for either 4 or 8 days. As shown in Figure 5.5, DHRS3 was strongly expressed in primary cultures decidualized for 4 and 8



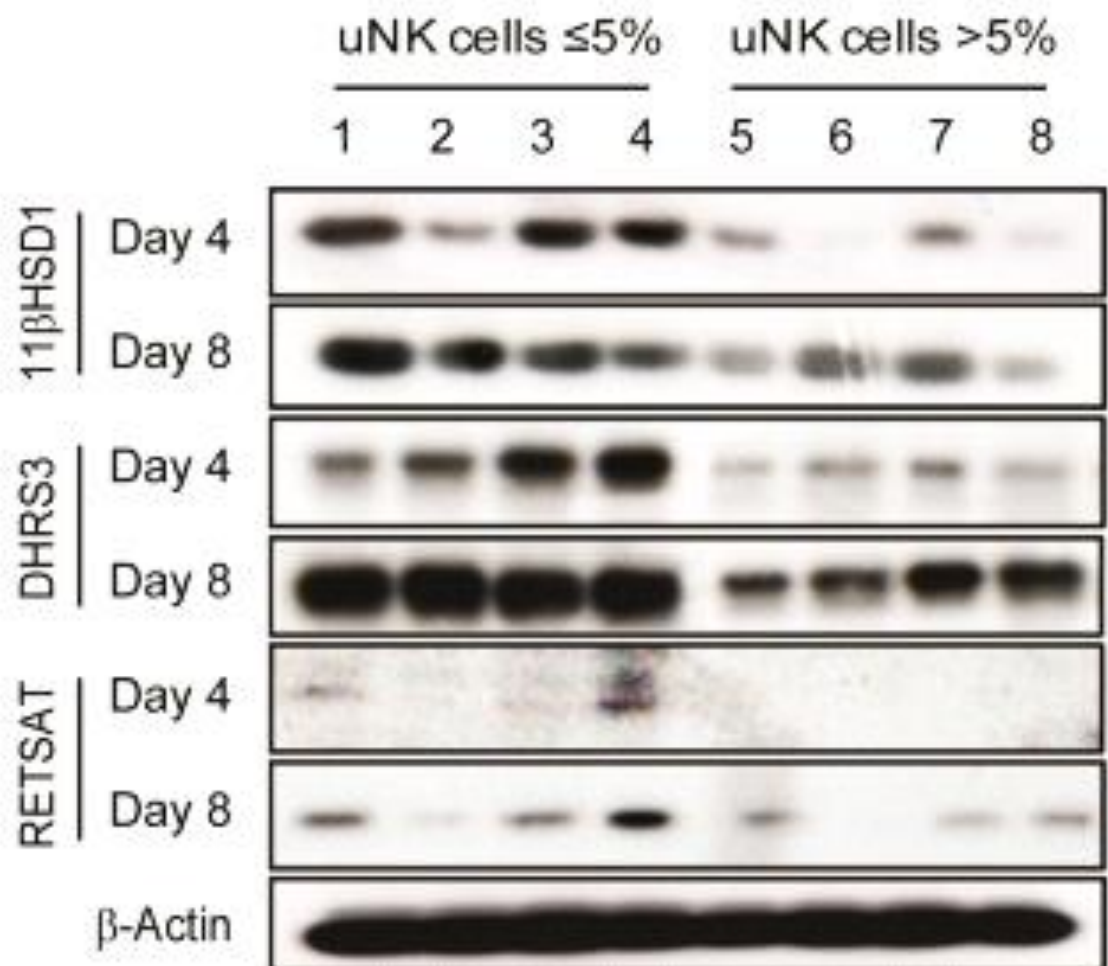
days and noticeably much more so in cultures established from samples with normal uNK cell density. By contrast, RETSAT was barely detectable after 4 days of differentiation in both sample sets, although by 8 days, expression appeared stronger in cultures derived from samples with 5% CD56+ cell densities. The overall pattern of DHRS3 and RETSAT expression in these time-course cultures correlated well with the expression of 11 $\beta$ HSD1 (Fig. 5.5).

**A****B**

**Figure 5.3:** Immunohistochemistry - GR and MR staining of undifferentiated HESCs. A. GR staining (brown stain) noted in the nucleus and cytoplasm of endometrial glandular cells as well as in the glandular lumen. Semi-quantitative analysis shown in the right did not show statistical significance between the low and high uNK cell groups. B. MR staining (brown stain) is seen in the nucleus and cytoplasm of endometrial stromal cells as well as in the glandular lumen. Semi-quantitative analysis shows higher concentration in the low uNK cell group, \*,  $P < 0.05$



**Figure 5.4:** The expression of DHR3 and RETSAT upon decidualization. **A** and **B** HESCs were untreated or treated with 8-bromo-cAMP, P4 and cortisone over a time course lasting 8 days. The DHR3 and RETSAT mRNA levels normalized to L19 are expressed as fold induction relative to expression levels in undifferentiated HESCs (dotted lines). The bar graphs (right panel) are analysed comprehensively from the relative expression data of DHR3 and RETSAT mRNA levels ( $\pm$ SEM) (left and centre panel), \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



**Figure 5.5:** 11βHSD1, DHRS3 and RETSAT protein expression in decidualizing endometrial cells depending on uNK cell count. This is composite figure showing 11βHSD1, DHRS3 and RETSAT protein expression in decidualizing endometrial cells on western blot analysis, and the correlation between the high and low uNK cell groups. β-Actin served as a loading controls

From the experiments detailed in chapter 3; we know that decidualizing stromal cells show significant increase in the expression of the enzyme 11 $\beta$  HSD1 (as opposed to 11 $\beta$ HSD2) which is primarily involved in the conversion of inactive glucocorticoid to the active form of glucocorticoid i.e. cortisol. Concurrently there is a shift from glucocorticoid dominance to mineralocorticoid dominance upon stromal cell decidualization. The experiments in chapter 4 draw us to the fact that high uNK cell density in the sub epithelial region of the endometrium is associated with impaired decidualization. The current chapter marries the above together with the finding that high uNK cell density down regulates 11 $\beta$ HSD1 expression. The expression of glucocorticoid receptors does not show much correlation with the uNK cell density; however, the mineralocorticoid receptors are down regulated in the endometrium with high uNK cell density. The association of high uNK cell density with defective decidualization and glucocorticoid Signalling was further supported by the finding of impaired induction of *DHRS3* and *RETSAT* transcripts in decidualizing cultures established from high uNK cell biopsies. These enzymes are involved in lipid metabolism and retinoid acid (RA) biosynthesis and storage. Both shortage and excess of RA contribute to fetal malformation, suggesting that retinoid metabolism must be regulated at the feto-maternal interface (Han *et al.*, 2010). Alcohol dehydrogenase and NADP(H)-dependent SDR, including DHRS3, oxidize retinal to retinol and promote its storage as retinyl esters. Similarly, RETSAT is involved in regulation of retinoid storage as lipid droplets (Schupp *et al.*, 2009). Interestingly, RA inhibits decidualization of HESCs and excess levels of RA or retinal are cytotoxic (Brar *et al.*, 1996). Thus, high uNK cell density

in the peri-implantation endometrium may be associated with perturbations in the retinoid metabolism pathway in the stromal compartment, which in turn predispose for an impaired decidual response and compromise histiotrophic support of the early conceptus.

Thus, endometrium with high uNK cell density as seen in women with recurrent miscarriage and recurrent implantation failure show aberrant decidualization with impaired induction of the 11 $\beta$ HSD1 which is the key enzyme in the local corticosteroid metabolism with a more significant impact on the mineralocorticoid dependant retinoid metabolism in these cells.

## LIMITATIONS OF THE STUDY:

The hypotheses of this study were derived from different strands of clinical evidence showing association between recurrent miscarriage and high uterine NK cell density in their endometrium. The uterine biopsies taken in the luteal phase of the menstrual cycle were analysed for the uterine NK cell density between days 7-10 after LH surge. Ideally, all biopsies should be timed on the same day i.e. LH + 7 days as previously published. However this was not possible due to logistic reasons due to biopsies falling over weekend. This has an impact on the uNK cell density as their numbers increase through the decidual phase from days 7 to 10 post LH surge.

The uNK cell density was analysed in the sub epithelial region of the endometrium at 40X magnification. Along the strip of endometrium stained for CD56; five high power fields are chosen and the uterine NK cell density counted and expressed as the percentage amongst stromal cells. There is a natural tendency to be drawn towards highly stained area of the sub epithelial region. This gives rise to the question whether this type of selection creates subjective bias. One could argue that it is in fact more pertinent that nests of high density stained areas are analysed which might be relevant in causing endometrial aberration in women with recurrent miscarriage or recurrent implantation failure. The depth to which these CD56 stained cells are analysed for quantification is yet to be determined. In order to minimise variations; in this study 40X magnification used with endometrial endothelium in view with minimal vacant spaces as possible. However, in this study, the same observer did the quantitative assessment and used the same standard

for all the samples. Due to this, the results are unlikely to change in their interpretation.

Secondly, the cut off taken to quantify the uNK cell density of 5% as high is based on some previous observational studies. One can notice in the experiments that this cut off is in fact difficult to confirm as there appears to be a few samples which lie between 5 and 10%. These samples do not quite show the marked differences in the induction of the key enzymes and target genes studied. This is more apparent when logarithmic scale is used to correlate the findings. The cut-off needs to be determined based on a larger population study. Again, in this study, the same observer did the quantification and used the same cut-off for all samples.

The cell culture studies undertaken are in vitro, hence not an exact replication of the in vivo state. In the molecular primary cell culture studies, the expression of 11 $\beta$ HSD1 was assessed by challenging it with the inactive cortisone as substrate for its activity. The significant induction of the expression of 11 $\beta$ HSD1 was good enough to imply that the 11 $\beta$ HSD1 produces the critical ligand(s) that controls GR- and MR-dependent gene expression in differentiating HESCs. Hence experiments to silence 11 $\beta$ HSD1 via siRNA administration and then assessing the impact of the loss of function on GR- and MR-dependent gene expression during decidualization were not done. This would have further helped bolster the hypothesis that ligands produced by 11 $\beta$ HSD1 indeed control the pathways regulated by GR and MR in human endometrium.



Time permitting, one could have further studied and determined that the downstream genes of GR or MR such as ZNF486 or GR1A1 are indeed the primary target of these receptors. This would involve bioinformatics analyses of selected genes to examine whether they harbour the genes in their promoter/enhancer regions. Also chromatin immunoprecipitation could be done to verify whether these genes are direct targets of GR or MR.

We also recognise the microarray analyses which yielded a number of genes coding for GR and MR are downstream genes and may not necessarily be the primary targets of these receptors.

Despite some of the limitations enumerated; this study gives a strong molecular basis to the clinical studies in women with recurrent miscarriage and recurrent implantation failure that:

- High uNK cell density is indeed associated with aberrant endometrial decidualization process in such women
- The possible role of progesterone supplement during the window of implantation and in the early implantation phase
- Role of corticosteroid as a possible immune modulatory treatment

These need to be validated in a larger randomised controlled trial. The proposed design for this trial is that of a prospective multi-centre randomised controlled trial where prednisolone versus placebo is used in the luteal and early implantation phase of women with recurrent miscarriage and high uNK cell counts.

## **Chapter 6: Conclusion**

Human reproduction is a relatively inefficient process with a probability of conception of 25–30% per cycle and only 50% of these conceptions advance beyond 20 weeks of gestation. Of the pregnancies that are lost, 75% represent a failure of implantation and are therefore not clinically recognized as pregnancies (Norwitz *et al.*, 2001). Recurrent Pregnancy Loss (RPL) is defined as the loss of three or more consecutive pregnancies, affects 1% of couples trying to conceive (Stirrat, 1990) It has been estimated that 1–2% of second-trimester pregnancies miscarry before 24 weeks of gestation (Wyatt *et al.*, 2005). RPL is a prevalent disorder that affects 1%–2% of couples and a cause of considerable physical and psychological morbidity (Rai & Regan, 2006). Early pregnancy loss is a frustrating and heart-wrenching experience for both the patient and the physician and poses a challenge to the clinicians and immunologists. Treatment of proven efficacy is available for only 15% of women with RM, e.g., those with antiphospholipid syndrome (Quenby *et al.*, 2005b). Several studies have tried to understand the aetiology of this distressing condition. Of these, reproductive immunology is a fast emerging concept especially in women with recurrent miscarriages allowing immunomodulation as treatment prospect. Uterine natural killer (NK) cells are the most studied in this field.

Human uterine endometrial natural killer (uNK) cells are the unique subset of peripheral NK cells, being positive for CD56+ and CD16-. They are unique in their location, function and phenotype. This subset of NK cells in the uterine endometrium is the example for regulatory function of NK cells as opposed to the cytotoxic function of the CD<sup>56</sup><sup>dim</sup> CD<sup>16</sup> positive NK cell subset in the

peripheral blood. Ablation of these cells in mice has been shown to compromise spiral arteriole remodelling and maintenance of decidual integrity seen after mid-pregnancy (Croy *et al.*, 2003). Yet uNK cell deficient IL15<sup>-/-</sup> mice are fertile, have normal gestation lengths and litter sizes comparable to wild-type mice (Barber & Pollard, 2003). Many roles have been implicated to the uNK cells including spiral artery remodelling, decidual angiogenesis and maternal allorecognition. They are a rich source of angiogenic growth factors. However, it is interesting to note that the endometrium of RPL patients is characterized by reduced expression of several key factors, including PDGF-BB, Ang-2, VEGF-A and VEGF-C (Lash *et al.*, 2012). Hence the function and mechanism of the roles of uNK cells still remain unclear.

Increased uNK cell density in mid-luteal endometrium has been associated with reproductive failure, especially RPL (Chazara *et al.*, 2011; Quenby *et al.*, 2009; Clifford *et al.*, 1999). However, the mechanisms that account for cyclic recruitment of uNK cell precursors and subsequent proliferation and differentiation within the peri-implantation environment are not well understood. In addition to IL-11 and IL-15, several other endometrial factors may be implicated in this process, including chemokine (C-X-C) motif ligand 14, IL-12 and IL-33 (Karsten *et al.*, 2009; Mokhtar *et al.*, 2010; Salker *et al.*, 2012). Human uNK cells have been described as immature and inactive before pregnancy (Manaster *et al.*, 2008). It is unclear as to how high uNK cell densities prior to conception predisposes for subsequent pregnancy failure.

Studies have shown that preconceptual prednisolone treatment markedly reduces uNK cell density in RPL patients (Quenby *et al.*, 2005a) and uterine NK cells express GR but not progesterone receptors (Guo *et al.*, 2012). These suggest that glucocorticoids are likely to act directly on these cells. We have seen that 11 $\beta$ HSD1 expression and enzyme activity is significantly up regulated in decidualizing HESCs and this is driven by cAMP and P4 signalling (Kuroda *et al.*, 2012). Further, inhibition of 11 $\beta$ HSD1 activity with either carbenoxolone disodium salt or PF 915275 virtually abolishes the induction of 11 $\beta$ HSD1, indicating that local cortisol signalling reinforces the expression of this enzyme in decidualizing cells (Kuroda *et al.*, 2012). Upon decidualization, there is a shift from glucocorticoid to mineralocorticoid dominance. The expression of MR related genes are up regulated upon decidualization. There appears to be an induction of cortisol gradient upon decidualization of HESCs. The experiments show a strong negative correlation between uNK cell densities and expression of 11 $\beta$ HSD1 in differentiating stromal cells *in vivo*. High uNK cell density *in vivo* is associated with blunted induction of 11 $\beta$ HSD1 as well as decidual marker genes, such as *PRL* and *IGFBP1*, in primary cultures. Upon studying the cytokines which are thought to have regulatory function on uNK cells, the induction of IL-11 and IL-15 also tended to be lower in decidualizing cells established from biopsies with elevated uNK cell levels, although this trend was not significant. The decidual process in the human endometrium is under tight spatiotemporal control (Gellersen *et al.*, 2007). It is initiated in the mid-luteal phase of the cycle first in stromal cells surrounding the terminal spiral arteries and underlying the luminal epithelium. Thus, rather than the

total number of uNK cells, it is possible that excessive migration of uNK cells from their usual position in the basal and peri-vascular regions of the endometrium to the sub-luminal region is the hallmark of an abnormal decidual response that predisposes for early pregnancy loss. Thus, increased density of CD56+ cells in the subluminal endometrial stromal compartment may be an indirect marker of local corticosteroid deficiency. We have seen that there is blunted induction of 11 $\beta$ HSD1, MR and MR related genes *DHRS3* and *RETSAT* transcripts in decidualizing cultures established from high uNK cell biopsies. These enzymes are involved in lipid metabolism and retinoid acid (RA) biosynthesis and storage. Both shortage and excess of RA contribute to foetal malformation, suggesting that retinoid metabolism must be regulated at the fetomaternal interface (Han *et al.*, 2010). RA inhibits decidualization of HESCs and excess levels of RA or retinal are cytotoxic (Brar *et al.*, 1996). Thus, high uNK cell density in the peri-implantation endometrium may be associated with perturbations in the retinoid metabolism pathway in the stromal compartment, which in turn predisposes for an impaired decidual response and compromise histiotrophic support of the early conceptus.

Thus, the spatiotemporal distribution of uNK cells is a complex and dynamic process and this is closely knitted with the human endometrial stromal cells. Besides glands and other immune cells, decidualizing stromal cells play a major role in governing this process, at least partially by inducing a cortisol gradient that establishes a nutritive environment essential for post-implantation embryo development and foetal growth. Thus excessive uNK cells in the subluminal stroma compartment prior to conception may serve as

a potential biomarker for a suboptimal decidual response in pregnancy. A recent pilot randomised, double-blind controlled clinical trial suggested an improvement in live birth rate with prednisolone in women with RPL and high mid-luteal uNK cell density (Tang *et al.*, 2011). Future study needs validating these results in a larger trial.







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## Appendix

# Appendix I

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## **Division of Obstetrics and Gynaecology**

### **Information for Women**

**Title of Project:** Research into the womb lining and fertility.

You are being invited to take part in a research study. Before you decide whether you wish to participate or not, it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and discuss it with relatives, friends and your GP, if you wish. If there is anything that is not clear, or if you have any further questions, please do not hesitate to ask. Take time to decide whether you would like to take part or not. You should not take part in this study if you do not wish to. If you decide not to take part, your treatment will not be affected by your decision. If you decide to participate, please let us know beforehand if you are currently involved in another study. You are free to withdraw at any time without explanation and any subsequent treatment will not be affected.

The womb lining sheds and regenerates once every month, when you have a period it is this lining coming away. The womb lining is the site where the embryo attaches and commences growing to become a pregnancy. We are doing research looking for reasons why the womb lining in some diseases may be less receptive to an embryo. This research involves testing for products (such as proteins) in the womb lining, which may be important for the embryo to attach.

You have been asked to take part in this study because you will be having an operation in a few days' time. The doctor's looking after you will be removing part of your womb lining as part of your treatment. A small part of the womb lining is removed from your womb while you are under anaesthetic. This does not cause you extra pain, discomfort or inconvenience. Normally, unless you request otherwise, your excess womb lining is disposed of once sufficient sample has been sent for diagnosis. However, if you agree to take part in the study, the doctor looking after you will ensure that the spare womb lining is passed to us for study in laboratory. Extra tissue will not be taken for the purposes of the research. There is no risk to you from donating some of your womb lining above that of the surgical procedure which you will soon undertake. It will leave no long term effects because your womb lining regenerates every month.

After you have donated the tissue, the researchers will not need to see you again and there is no need for you to visit the GP. Although there is no immediate medical benefit to you from taking part, the information that we get from this study may help us to treat women in the future who experience problems with infertility.



Women who are pregnant cannot take part in this study and those at risk of a pregnancy will have a pregnancy test performed to exclude the possibility of pregnancy.

The researchers working on these samples will not know which samples belong to which women, as they will have been coded (given a reference number) and there will be no way of tracing the samples back to individual donors. It is possible that the results from the research study could be published in a medical or scientific journal. However, please be reassured that you will not be identified in any such publication. Confidentiality of data is therefore assured.

If you do suffer any adverse effects because of your participation in this study, you will be compensated through the University Hospitals of Coventry and Warwickshire, "No Fault" Compensation Scheme.

For further information, please contact:

Professor Jan Brosens on 0247696 8704 (email: [J.J.Brosens@warwick.ac.uk](mailto:J.J.Brosens@warwick.ac.uk))

Thank you for taking part in this study. You will be given a copy of the information sheet to keep.

(Version 00.2:18/11/2009)

**Sample Collection, Contact & Clinical Details****SAMPLE COLLECTION:**

- Obtain endometrial sample by pipelle or curette (more= better)
- Put sample in sterilin with normal saline or culture medium
- Organize transport to lab.

**CONTACT DETAILS:**

Prof Jan Brosens: 0247696 8704

Lab Number: 0247696 8612

**PATIENT DETAILS (Please complete)**

Age:.....

Recurrent miscarriage: Y/N

Time-to-pregnancy: <3months, <6months, >6months (please circle)

Infertility: Y/N

Endometriosis/tubal/male factor/unexplained/PCOS (please circle)

Other:.....

LMP:.....

Parity:.....

Current treatment:.....

**Participant Consent Form****Title of project:**

Establishment of primary cell cultures using biopsy samples of human endometrium as a model to investigate decidualization and implantation.

The participant should complete the whole of this sheet him or herself.

(Please tick each statement if it applies to you)

I have read the Information sheet for Patients and Healthy Volunteers. ☐

I have been given the opportunity to ask questions and discuss this study . ☐

I have received satisfactory answers to all my questions. ☐

I have received enough information about the study. ☐

The study has been explained to me by:

Prof/Dr/Mr/Mrs/Ms.....  
.....

I understand that I am free to withdraw from the study  
at any time, without having to give a reason for  
withdrawing and without affecting my future medical care. ☐

I agree to take part in this study. ☐

Signed.....Date.....  
.....

Name in Block  
Capitals.....

Investigator's  
signature.....Date.....

Name in Block Capitals.....

## Appendix II

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### RTQ-PCR primer pairs

Gene	Forward Primer	Reverse Primer
L19	GCG GAA GGG TAC AGC CAA	GCA GCC GGC GCA AA
11 $\beta$ HSD 1	AGC AAG TTT GCT TTG GAT GG	AGA GCT CCC CCT TTG ATG AT
GR	CCC TAC CCT GGT GTC ACT GT	GGT CAT TTG GTC ATC CAG GT
MR	GGC ACT CGC TGG CCT GGA TG	GTC TCC ATC GCT GCC TCG GC
PRL	AAG CTG TAG AGA TTG AGG AGC AAA C	TCA GGA TGA ACC TGG CTG ACT A
IGFBP1	CGAAGG CTC TCC ATG TCA CCA	TGT CTC CTG TGC CTT GGC TAA AC
WNT 4	TCA GCC CAC AGG GCT TCC AGT	CGG CTC GCC AGC ACG TCT
DHRS3	AGC GCG GCG CCA GAA AGA TT	TCA CCC ACC TTC TCC CGG ACG
FBXO32	GCG GCA GTT TCG TGA GCG AC	GGG TGC AAT ATC CAT GGC GCT CTT
GRIA1	AAG GGG TCT GCC CTG AGA AAT CCA	ACG CCT GCC ACA TTG CTG AGG
ZNF486	ACA CAC AAC TGG GGG TTT GGC AAT	ACA CAC AAC TGG GGG TTT GGC AAT

# Appendix III

## Gene Lists

**Table 1:**

<b>Gene symbol</b>	<b>Gene name</b>	<b>Fold change</b>
SNORD41	Small nucleolar RNA, C/D box 41	-2.73
SNORD57	Small nucleolar RNA, C/D box 57	-2.71
A2M	Alpha-2-macroglobulin	-2.56
STARD5	StAR-related lipid transfer (START) domain containing 5	-2.46
SNORD75	Small nucleolar RNA, C/D box 75	-2.45
SMAP2	Small ArfGAP2	-2.43
SNORD15A	Small nucleolar RNA, C/D box 15A	-2.12
DHDDS	Dehydrodolichyl diphosphate synthase	-2.10
SERPINA3	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	-2.00
EIF5AL1	Eukaryotic translation initiation factor 5A-like 1	-1.92
BCAS3	Breast carcinoma amplified sequence 3	-1.86
AIG1	Androgen-induced 1	-1.82
SNORD116-6	Small nucleolar RNA, C/D box 116-6	-1.80
FECH	Ferrochelatase	-1.79
FAM86B1	Family with sequence similarity 86, member B1	-1.79
C9orf116	Chromosome 9 open reading frame 116	-1.79
DPCD	Deleted in primary ciliary dyskinesia homolog	-1.77
STARD9	StAR-related lipid transfer (START) domain containing 9	-1.76
LRCH1	Leucine-rich repeats and calponin homology (CH) domain	-1.74
ELOVL1	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 1	-1.74
HIST2H2AB	Histone cluster 2, H2ab	-1.73
HDAC3	Histone deacetylase 3	-1.71
SNX12	Sorting nexin 12	-1.71
ARHGAP1	Rho GTPase activating protein 1	-1.69
ENTPD3	Ectonucleoside triphosphate diphosphohydrolase 3	-1.69
MLXIP	MLX interacting protein	-1.69
<b>FBXO32</b>	F-box protein 32	-1.68
MAK16	MAK16 homolog	-1.68
MFSB8	Major facilitator superfamily domain containing 8	-1.67
ZNF528	Zinc finger protein 528	-1.67
ACSS3	Acyl-CoA synthetase short-chain family member 3	-1.64
LONP1	Lon peptidase 1, mitochondrial	-1.64
WDR31	WD repeat domain 31	-1.63
NAT10	N-acetyltransferase 10	-1.63
KRT23	Keratin 23 (histone deacetylase inducible)	-1.63
DHRS4	Dehydrogenase/reductase (SDR family) member 4	-1.62
LIMK1	LIM domain kinase 1	-1.61
C1QTNF1	C1q and tumor necrosis factor related protein 1	-1.60
MIPEP	Mitochondrial intermediate peptidase	-1.60
DONSON	Downstream neighbor of SON	-1.59
GNAL	Guanine nucleotide binding protein (G protein), alpha activating activity polypeptide, olfactory type	-1.59
ADCY2	Adenylate cyclase 2	-1.59
TEX261	Testis expressed 261	-1.58
C1QTNF9	C1q and tumor necrosis factor related protein 9	-1.58
GYPE	Glycophorin E	-1.58
MRPL40	Mitochondrial ribosomal protein L40	-1.57
ATF1	Activating transcription factor 1	-1.56
RETSAT	Retinol saturase (all-trans-retinol 13,14-reductase)	-1.54
STK16	Serine/threonine kinase 16	-1.53
<b>DHRS3</b>	Dehydrogenase/reductase (SDR family) member 3	-1.51

**Table 2. Genes up-regulated upon MR knockdown**

<b>Gene symbol</b>	<b>Gene name</b>	<b>Fold change</b>
LRRFIP1	Leucine rich repeat (in FLII) interacting protein 1	2.49
LRRC37B	Leucine rich repeat containing 37B	2.07
NUFIP1	Nuclear fragile X mental retardation protein interacting protein 1	2.06
C7orf13	Chromosome 7 open reading frame 13	2.04
MAP3K1	Mitogen-activated protein kinase kinase kinase 1	1.97
RTP4	Receptor (chemosensory) transporter protein 4	1.93
NAG18	NAG18 Mrna	1.77
LOC100128364	Hypothetical protein LOC100128364	1.77
MURC	Muscle-related coiled-coil protein	1.75
WDSUB1	WD repeat, sterile alpha motif and U-box domain containing 1	1.75
FAM19A2	Family with sequence similarity 19 (chemokine (C-C motif)-like), member A2	1.72
GCNT4	Glucosaminyl (N-acetyl) transferase 4, core 2	1.71
SYDE2	Synapse defective 1, Rho GTPase, homolog 2	1.70
FAM99A	Family with sequence similarity 99, member A	1.69
TRMU	tRNA 5-methylaminomethyl-2-thiouridylate methyltransferase	1.67
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1	1.66
ZNF223	Zinc finger protein 223	1.65
ZCCHC14	Zinc finger, CCHC domain containing 14	1.65
SNORD45B	Small nucleolar RNA, C/D box 45B	1.63
CCRL1	Chemokine (C-C motif) receptor-like 1	1.61
FAM106A	Family with sequence similarity 106, member A	1.60
DPH3B	DPH3B, KTI11 homolog B	1.59
LOC220594	TL132 protein	1.58
ZNF181	Zinc finger protein 181	1.58
LCN8	Lipocalin 8	1.58
SGK3	Serum/glucocorticoid regulated kinase family, member 3	1.56
SLITRK6	SLIT and NTRK-like family, member 6	1.56
CLN8	Ceroid-lipofuscinosis, neuronal 8	1.55
ID2	Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein	1.54
LOC100129455	Hypothetical protein LOC100129455	1.54
MMD	Monocyte to macrophage differentiation-associated	1.53
C20orf177	Chromosome 20 open reading frame 177	1.52
SLC25A2	Solute carrier family 25	1.51
HDX	Highly divergent homeobox	1.51
UPRT	Uracil phosphoribosyltransferase (FUR1) homolog	1.51

**Table 3. Top 50 down-regulated genes upon GR knockdown**

<b>Gene symbol</b>	<b>Gene name</b>	<b>Fold change</b>
SNORD74	Small nucleolar RNA, C/D box 74	-3.36
C13orf29	Chromosome 13 open reading frame 29	-2.33
ADAMTS2	ADAM metalloproteinase with thrombospondin type 1 motif, 2	-2.25
STIM1	Stromal interaction molecule 1	-1.90
ADCY9	Adenylate cyclase 9	-1.83
NRSN2	Neurensin 2	-1.83
SLC25A20	Solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	-1.83
ACSS2	Acyl-CoA synthetase short-chain family member 2	-1.81
TIMM22	Translocase of inner mitochondrial membrane 22 homolog	-1.76
SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	-1.73
EIF3B	Eukaryotic translation initiation factor 3, subunit B	-1.71
ACSL1	Acyl-CoA synthetase long-chain family member 1	-1.71
FAM48B2	Family with sequence similarity 48, member B2	-1.71
PMM1	Phosphomannomutase 1	-1.70
ACACA	Acetyl-CoA carboxylase alpha	-1.68
FAM164C	Family with sequence similarity 164, member C	-1.65
TANC2	Tetratricopeptide repeat, ankyrin repeat and coiled-coil containing 2	-1.64
NSMCE1	Non-SMC element 1 homolog	-1.64
RHOC	Ras homolog gene family, member C	-1.64
P4HA3	Prolyl 4-hydroxylase, alpha polypeptide III	-1.63
AMFR	Autocrine motility factor receptor	-1.63
TSPAN5	Tetraspanin 5	-1.63
FAM50A	Family with sequence similarity 50, member A	-1.63
AIFM2	Apoptosis-inducing factor, mitochondrion-associated, 2	-1.62
GNA11	Guanine nucleotide binding protein (G protein), alpha 11	-1.62
SURF4	surfeit 4	-1.62
LRPAP1	low density lipoprotein receptor-related protein associated protein 1	-1.62
TLN1	Talin 1	-1.61
DEPDC6	DEP domain containing 6	-1.61
SNF8	SNF8, ESCRT-II complex subunit, homolog	-1.61
VAV2	Vav 2 guanine nucleotide exchange factor	-1.60
SNX17	Sorting nexin 17	-1.60
ANPEP	Alanyl (membrane) aminopeptidase	-1.60
YRDC	YrdC domain containing	-1.59
ACTC1	Actin, alpha, cardiac muscle 1	-1.59
MMAB	Methylmalonic aciduria (cobalamin deficiency) cblB type	-1.59
LMNA	Lamin A/C	-1.59
TBC1D2B	TBC1 domain family, member 2B	-1.59
EYS	Eyes shut homolog	-1.58
SDCCAG1	Serologically defined colon cancer antigen 1	-1.58
RABAC1	Rab acceptor 1 (prenylated)	-1.58
NDUFV1	NADH dehydrogenase (ubiquinone) flavoprotein 1	-1.58
FTSJ2	FtsJ methyltransferase domain containing 2	-1.58
WNT4	Wingless-type MMTV integration site family, member 4	-1.57
OGDH	Oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	-1.57
R3HCC1	R3H domain and coiled-coil containing 1	-1.57
APEH	N-acylaminoacyl-peptide hydrolase	-1.56
RASA3	RAS p21 protein activator 3	-1.56
PLOD1	Procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1	-1.56
ABR	Active BCR-related gene	-1.56

**Table 4. Top 50 up-regulated genes upon GR knockdown**

<b>Gene symbol</b>	<b>Gene name</b>	<b>Fold change</b>
SNORD13P2	Small nucleolar RNA, C/D box 13 pseudogene 2	3.60
LOC100130876	Hypothetical protein LOC100130876	3.26
<b>ZNF486</b>	Zinc finger protein 486	2.49
GPAM	Glycerol-3-phosphate acyltransferase, mitochondrial	2.45
TUBD1	Tubulin, delta 1	2.37
HAS2	Hyaluronan synthase 2	2.35
HMGN5	High-mobility group nucleosome binding domain 5	2.19
MCM9	Minichromosome maintenance complex component 9	2.18
SPP1	Secreted phosphoprotein 1 (Osteopontin)	2.14
ZNF791	Zinc finger protein 791	2.13
RRP7B	Ribosomal RNA processing 7 homolog B	2.07
RGS4	Regulator of G-protein Signalling 4	2.06
SNORA75	Small nucleolar RNA, H/ACA box 75	2.05
UPF3B	UPF3 regulator of nonsense transcripts homolog B	2.00
NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2	1.99
PMS1	PMS1 postmeiotic segregation increased 1	1.95
C14orf106	Chromosome 14 open reading frame 106	1.92
RWDD4A	RWD domain containing 4A	1.91
ZNF738	Zinc finger protein 738	1.90
ZNF253	Zinc finger protein 253	1.88
CFI	Complement factor I	1.86
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	1.84
ATXN3	Ataxin 3	1.83
ING2	Inhibitor of growth family, member 2	1.82
ZNF613	Zinc finger protein 613	1.80
COX7B	Cytochrome c oxidase subunit VIIb	1.79
EIF1B	Eukaryotic translation initiation factor 1B	1.78
ZNF626	Zinc finger protein 626	1.78
NCRNA00204	Non-protein coding RNA 204	1.76
ZNF311	Zinc finger protein 311	1.74
CCDC28A	Coiled-coil domain containing 28A	1.73
LYRM1	LYR motif containing 1	1.72
ARID4B	AT rich interactive domain 4B (RBP1-like)	1.72
RAC1	Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)	1.72
NKAP	NFKB activating protein	1.71
ZNF91	Zinc finger protein 91	1.71
SEPP1	Selenoprotein P, plasma, 1	1.71
PALMD	Palmdelphin	1.70
ZNF624	Zinc finger protein 624	1.70
GCC2	GRIP and coiled-coil domain containing 2	1.70
RBAK	RB-associated KRAB zinc finger	1.70
PPIL4	Peptidylprolyl isomerase (cyclophilin)-like 4	1.69
SET	SET nuclear oncogene	1.69
RIPK2	Receptor-interacting serine-threonine kinase 2	1.67
MTF2	Metal response element binding transcription factor 2	1.67
ZNF420	Zinc finger protein 420	1.66
NCRNA00219	Non-protein coding RNA 219	1.66
RFC1	Replication factor C (activator 1) 1	1.65
EXTL2	Exostoses (multiple)-like 2	1.65
NMI	N-myc (and STAT) interactor	1.65



**Table 5. Genes regulated by MR and GR**

<b>Gene symbol</b>	<b>Gene name</b>	<b>MR siRNA fold change</b>	<b>GR siRNA fold change</b>
C15orf51	Dynamin 1 pseudogene	-5.82	-3.24
LOC100289668	Similar to dynamin 1	-4.72	-3.21
SNORD14E	Small nucleolar RNA, C/D box 14E	-4.71	-3.10
SNORD4B	Small nucleolar RNA, C/D box 4B	-3.52	-3.05
SNORA73A	Small nucleolar RNA, H/ACA box 73A	-3.19	-1.79
MIR29C	MicroRNA 29c	-2.26	-1.93
SNORD82	Small nucleolar RNA, C/D box 82	-2.20	-2.04
SNORD38B	Small nucleolar RNA, C/D box 38B	-2.17	-2.15
TMEM194B	Transmembrane protein 194B	-2.14	-1.83
SCARNA17	Small Cajal body-specific RNA 17	-2.02	-1.87
MGC16384	Hypothetical protein LOC114130	-1.95	-2.23
HFE	Hemochromatosis	-1.94	-1.75
NOP56	NOP56 ribonucleoprotein homolog	-1.93	-1.51
LARGE	Like-glycosyltransferase	-1.86	-2.02
ENO1	Enolase 1, (alpha)	-1.84	-1.63
SRGAP2	SLIT-ROBO Rho GTPase activating protein 2	-1.79	-1.66
PARP3	Poly (ADP-ribose) polymerase family, member 3	-1.77	-1.64
DHCR7	7-dehydrocholesterol reductase	-1.74	-1.60
NUDT8	Nudix (nucleoside diphosphate linked moiety X)- type motif 8	-1.74	-1.53
IFI35	Interferon-induced protein 35	-1.72	-1.70
RCAN3	RCAN family member 3	-1.70	-1.54
CCDC88C	Coiled-coil domain containing 88C	-1.68	-1.65
ATPAF2	ATP synthase mitochondrial F1 complex assembly factor 2	-1.67	-1.52
LSAMP	Limbic system-associated membrane protein	-1.67	-1.76
MPPE1	Metallophosphoesterase 1	-1.67	-1.72
LOC100132356	Hypothetical protein LOC100132356	-1.63	-1.51
ALKBH2	alkB, alkylation repair homolog 2	-1.62	-1.74
CD81	CD81 molecule	-1.62	-1.51
DHRS4L2	Dehydrogenase/reductase (SDR family) member 4 like 2	-1.61	-1.51
SNORA70G	Small nucleolar RNA, H/ACA box 70G (retrotransposed)	-1.59	-1.59
CD151	CD151 molecule (Raph blood group)	-1.59	-1.56
YIF1A	Yip1 interacting factor homolog A	-1.58	-1.54
STX17	Syntaxin 17	-1.57	-1.56
CCDC149	Coiled-coil domain containing 149	-1.56	-1.66
ANXA6	Annexin A6	-1.55	-1.78
DNM2	Dynamin 2	-1.55	-1.57
<b>GRIA1</b>	Glutamate receptor, ionotropic, AMPA 1	-1.54	1.77
KRT19	Keratin 19	-1.54	-1.65
KDM4C	Lysine (K)-specific demethylase 4C	-1.53	-1.69
LITAF	Lipopolysaccharide-induced TNF factor	-1.51	-1.58
MYO1C	Myosin IC	-1.51	-1.62
PRKACA	Protein kinase, cAMP-dependent, catalytic, alpha	-1.51	-1.50
ZNF254	Zinc finger protein 254	1.50	1.57
TFPI	Tissue factor pathway inhibitor (lipoprotein- associated coagulation inhibitor)	1.50	1.57
YEATS4	YEATS domain containing 4	1.51	1.56
HDGFRP3	Hepatoma-derived growth factor, related protein 3	1.57	1.55
IKZF5	IKAROS family zinc finger 5	1.58	1.57
PCDHB16	Protocadherin beta 16	1.58	1.50
CCDC14	Coiled-coil domain containing 14	1.58	1.64
ZNF625	Zinc finger protein 625	1.60	1.55
TAF1A	TATA box binding protein (TBP)-associated factor, RNA polymerase I, A	1.62	1.51
C17orf51	Chromosome 17 open reading frame 51	1.71	1.74
ZNF669	Zinc finger protein 669	1.76	1.78
SNORD73A	Small nucleolar RNA, C/D box 73A	1.76	2.17
AMZ2P1	Archaelysin family metallopeptidase 2 pseudogene 1	1.79	1.82
SASS6	Spindle assembly 6 homolog	1.83	2.05
ARL15	ADP-ribosylation factor-like 15	1.96	1.98

LOC100130713	Hypothetical protein LOC100130713	2.25	1.79
FBXO9	F-box protein 9	2.42	3.15
LOC729595	Similar to high mobility group box 3	3.01	4.64

**Table 6. MR / GR genes also regulated by AR or PR**

Gene symbol	Gene name	MR	GR	AR	PR
WDSUB1	WD repeat, sterile alpha motif and U-box domain containing 1	↑	—	↓	—
HDGFRP3	Hepatoma-derived growth factor, related protein 3	↑	↑	—	↑
LARGE	Like-glycosyltransferase	↓	↓	—	↓
MPPE1	Metallophosphoesterase 1	↓	↓	—	↓
NUDT8	Nudix (nucleoside diphosphate linked moiety X)-type motif 8	↓	↓	—	↓
ACSL1	Acyl-CoA synthetase long-chain family member 1	—	↓	↑	↓
SDCCAG1	Serologically defined colon cancer antigen 1	—	↓	↓	—
WNT4	Wingless-type MMTV integration site family, member 4	—	↓	↓	↓
ANPEP	Alanyl (membrane) aminopeptidase	—	↓	—	↓
DEPDC6	DEP domain containing 6	—	↓	—	↓
ZBTB1	Zinc finger and BTB domain containing 1	—	↑	↓	—
UPF3B	UPF3 regulator of nonsense transcripts homolog B	—	↑	—	↑

## Appendix IV: Publications

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- **Kuroda K\*, Venkatakrishnan R\***, James S, et al. Elevated periimplantation uterine natural killer cell density in human endometrium is associated with impaired corticosteroid signaling in decidualizing stromal cells. *J Clin Endocrinol Metab* 2013; **98** (11): 4429-37.
- Kuroda K, **Venkatakrishnan R**, Salker MS, et al. Induction of 11 $\beta$ -HSD 1 and activation of distinct mineralocorticoid receptor- and glucocorticoid receptor-dependent gene networks in decidualizing human endometrial stromal cells. *Mol Endocrinol* 2013; **27** (2): 192-202.

## Elevated Periimplantation Uterine Natural Killer Cell Density in Human Endometrium Is Associated With Impaired Corticosteroid Signaling in Decidualizing Stromal Cells

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**Background:** Decidualizing human endometrial stromal cells (HESCs) profoundly up-regulate 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1), the enzyme that converts inert cortisone to active cortisol. We postulated that the induction of a cortisol gradient upon decidualization of the periimplantation endometrium may impact on the uterine natural killer (uNK) cell population and on local expression of corticosteroid-dependent target genes.

**Methods:** Midluteal endometrial biopsies (n = 55) were processed for uNK cell (CD56) analysis and primary HESC cultures. The cultures remained either untreated or were decidualized for 4 or 8 days. A tissue microarray was constructed from endometria with normal (n = 18) and elevated uNK cell (n = 18) scores. An abnormal uNK cell test was defined as greater than 5% CD56<sup>+</sup> cells in the subluminal stroma.

**Results:** Increased uNK cell density was associated with lower endometrial expression of 11 $\beta$ HSD1 and mineralocorticoid receptor (MR) but not glucocorticoid receptor in vivo. Elevated uNK cell density also corresponded to impaired induction of key decidual markers (11 $\beta$ HSD1, prolactin, and insulin-like growth factor binding protein-1) and MR-dependent enzymes (dehydrogenase/reductase member 3 and retinol saturase) in differentiating HESC cultures. Increased uNK cell density in vivo was not associated with increased in vitro expression of either IL-15 or IL-11, two cytokines implicated in uNK cell regulation.

**Conclusions:** Elevated levels of uNK cells in the stroma underlying the surface epithelium are associated with inadequate cortisol biosynthesis by resident decidualizing cells and suboptimal induction of key MR-dependent enzymes involved in lipid biogenesis and the retinoid transport pathway. Our observations suggest that uNK cell testing identifies those women at risk of reproductive failure due to relative uterine cortisol deficiency. (*J Clin Endocrinol Metab* 98: 4429–4437, 2013)

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Abbreviations: DHRS, dehydrogenase/reductase; 8-bromo-cAMP, 8-bromoadenosine-cAMP; E, cortisone; GR, glucocorticoid receptor; HESC, human endometrial stromal cell; 11 $\beta$ HSD1, 11 $\beta$ -hydroxysteroid dehydrogenase; IGFBP1, IGF-binding protein-1; MR, mineralocorticoid receptor; P4, progesterone; PRL, prolactin; RA, retinoic acid; RETSAT, retinol saturase; RPL, recurrent pregnancy loss; TMA, tissue microarray; uNK, uterine natural killer.

Uterine natural killer (uNK) cells, an important component of the innate immune system, are the most abundant immune cells in midluteal (periimplantation) endometrium and in the decidua of early pregnancy (1). They represent a unique subset of natural killer cells, staining intensely for CD56 but not for CD16 antigens. uNK cells play a significant role in the establishment and maintenance of early pregnancy by promoting decidual angiogenesis, spiral artery remodeling, and trophoblast invasion (2, 3). In contrast to their circulating (CD56<sup>+</sup>/CD16<sup>+</sup>) counterparts, there is little evidence for a cytotoxic role of uNK cells at the fetomaternal interface. However, uNK cells express killer cell immunoglobulin-like receptors that preferentially bind to human leukocyte antigen-C molecules expressed on placental cells, suggesting a role in maternal allorecognition of fetal trophoblast (4). They are abundant around the spiral arteries, near endometrial glands, and adjacent to extravillous trophoblast in early pregnancy. Thus, uNK cells are unique in terms of their tissue distribution, phenotype, and function.

Both the maternal killer cell immunoglobulin-like receptor and fetal human leukocyte antigen-C gene systems are highly polymorphic and certain genotypic combinations are associated with a modest increase or decrease in pregnancy complications, including miscarriage, fetal growth restriction, and preeclampsia (4). In addition, several studies reported an association between elevated uNK cell levels in midluteal endometrium and reproductive failure (4–6). In particular, there is compelling evidence to link increased uNK density to recurrent pregnancy loss (RPL), defined here as three or more consecutive miscarriages. RPL is a prevalent disorder that affects 1%–2% of couples and a cause of considerable physical and psychological morbidity (7). Furthermore, RPL is associated with an increased likelihood of obstetric complications and adverse perinatal outcome in a subsequent ongoing pregnancy (8). Whether midluteal uNK cell testing in a non-conception cycle predicts subsequent pregnancy complications remains unresolved (9).

Resident human endometrial stromal cells (HESCs) are thought to serve as gatekeepers for the recruitment and distribution of immune cells in the periimplantation endometrium (10). For example, decidualizing (differentiating) HESCs secrete IL-11 and IL-15, two multifaceted cytokines implicated in trafficking and differentiation of uNK cells (11–13). uNK cells express the glucocorticoid receptor (GR) but lack progesterone receptor, rendering them directly responsive to cortisol but not progesterone (14, 15). Consistent with this notion, preconceptual glucocorticoid (prednisolone) treatment significantly reduces uNK cell density in RPL subjects as well as inhibiting endometrial angiogenesis (16, 17). We recently demon-

strated that progesterone massively enhances the expression and activity of 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1) in decidualizing HESCs (18), suggesting that local cortisol biosynthesis plays an integral role in the preparation of the endometrium for implantation. Decidualization is further associated with a decline in GR expression and reciprocal induction of the mineralocorticoid receptor (MR), which in turn drives the expression of several key enzymes involved in lipid and retinoid metabolism, including retinol saturase (RETSAT), various short-chain dehydrogenases/reductases (such as dehydrogenase/reductase (DHRS) member 3, DHRS4, and DHRS4L2), and steroidogenic acute regulatory protein-related lipid transfer protein 5 (18).

Emerging evidence suggests that aberrant differentiation of resident HESCs into specialized decidual cells is the hallmark of RPL (19, 20). Taken together, these observations raise the possibility that excessive uNK cell levels in midluteal endometrial samples reflect relative local corticosteroid deficiency, caused by inadequate induction of decidual 11 $\beta$ HSD1 and result in impaired local metabolic function.

## Materials and Methods

### Patient selection and endometrial sampling

The study was approved by the local ethics committee (1997/5065). Subjects were recruited in the Implantation Clinic, a dedicated research clinic at University Hospitals Coventry and Warwickshire National Health Service Trust for patients suffering RPL or recurrent in vitro fertilization treatment failure. Written informed consent was obtained prior to tissue collection. Endometrial biopsies were timed between 7 and 10 days after the preovulatory LH surge. Samples were obtained using a Wallach Endocell sampler (Wallach) under ultrasound guidance, starting from the uterine fundus and moving downward to the internal cervical os. Each biopsy was divided, with one part fixed in formalin for immunohistochemistry and the other processed for primary cell culture. The demographic details of participating subjects are summarized in Supplemental Tables 1 and 2, published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>.

### Primary cell culture

HESCs were isolated, cultured, and maintained as described (20). Primary cultures were passaged once, allowed to grow to confluency, and then decidualized with 0.5 mM 8-bromoadenosine cAMP (8-bromo-cAMP; Sigma), 1  $\mu$ M progesterone (P4; Sigma), and 0.1  $\mu$ M cortisone (E; Sigma). Cortisone, which is inactive, was added to decidualizing HESC cultures as the substrate for endogenous conversion by 11 $\beta$ HSD11 to cortisol (18).

## Immunohistochemistry

Five-micrometer-thick formalin-fixed, paraffin-embedded tissue sections were labeled with antibody to CD56 (NCL-CD56-1B6; Novocastra) using standard methods and detection systems (3). The uNK cell density was determined as the percentage of uNK cells within the stromal cell population. Because uNK cell density varies with endometrial depth, counting of CD56+ cells was confined to the stroma underlying the luminal epithelium. Five randomly selected high-power magnification fields per biopsy were assessed using ImageJ software (Rasband, W. S., ImageJ, National Institutes of Health) to minimize inter-observer variability (21, 22). Normal uNK cell density was defined as 5% or less CD56+ cells in the stroma underlying the luminal epithelium (17, 23). A Mirax Midi slide scanner was used to scan bright-field sections with a  $\times 20$  objective with a resolution of 0.23  $\mu\text{m}/\text{pixel}$ . This produces images that can be dynamically manipulated within the viewer software, allowing optical magnifications up to  $\times 20$  and digital magnification to  $\times 200$ .

## Tissue microarray (TMA)

Areas of interest, ie, subepithelial regions, were spotted and tissue microarrays comprising duplicate 0.6-mm cores from 18 cases in each group were constructed using Alphelys TMA Designer R 2 version 1.0.0.8. Sections (3  $\mu\text{m}$ ) were cut from completed array blocks and transferred to silanized glass slides. Sections from these arrays were then stained for CD56, 1:200 (NCL-CD56-1B6; Novocastra); 11 $\beta$ HSD1, 1:300 (AB83522; Abcam); MR, 1:400 (H-300; SC-11412; Santa Cruz Biotechnology); and GR, 1:200 (E-20; SC-1003; Santa Cruz Biotechnology). Semiquantitative analysis was performed using a Panoramic viewer to capture images. High-power ( $\times 400$ ) images were analyzed with Image J (<http://rsbweb.nih.gov/ij/>) using the color deconvolution plugin and thresholding to assess the percentage of strongly immunopositive endometrial cells. The observers were blind as to the origin of the samples.

## Real-time quantitative PCR

Total RNA was extracted with RNA STAT-60 from primary HESC cultures. After treatment with amplification-grade deoxyribonuclease I (Invitrogen Ltd), cDNA was generated using the SuperScript II first-strand synthesis system for RT-PCR kit (Invitrogen). Template quantification was performed with an ABI Step One system (Applied Biosystems) using Power SYBR Green PCR master mix (Applied Biosystems). RNA input variances were normalized against the levels of the *L19* housekeeping gene, which encodes a ribosomal protein. All measurements were performed in duplicate. Specific primer pairs were designed using Primer3 software (<http://frodo.wi.mit.edu/>): *L19* sense, 5'-GCG GAA GGG TAC AGC CAA T-3', *L19*-R antisense, 5'-GCA GCC GGC GCA AA-3'; 11 $\beta$ HSD1 sense, 5'-AGC AAG TTT GCT TTT GAT GG-3', 11 $\beta$ HSD1 antisense, 5'-AGA GCT CCC CCT TTG ATG AT-3'; decidual prolactin (PRL) sense, 5'-AAG CTG TAG AGA TTG AGG AGC AAA C-3', decidual PRL antisense, 5'-TCA GGA TGA ACC TGG CTG ACT A-3'; IGF-binding protein-1 (IGFBP1) sense, 5'-CGA AGG CTC TCC ATG TCA CCA-3', IGFBP1 antisense, 5'-TGT CTC CTG TGC CTT GGC TAA AC-3'; IL-11 sense, 5'-CTC GAG TTT CCC CAG ACC CTC GG-3', IL-11 antisense, 5'-TGT CAG CAC ACC TGG GAG CTG TAG-3'; IL-15 sense, 5'-TGG CTG CTG GAA ACC CCT TGC-3', IL-15 antisense, 5'-CCC TGC ACT

GAA ACA GCC CAA AA-3'; DHRS3 sense, 5'-AGC GCG GCG CCA GAA AGA TT-3', DHRS3 antisense, 5'-TCA CCC ACC TTC TCC CGG ACG-3'; and RETSAT sense, 5'-CGC TGC CTG CCA GGT GTG AAG-3', RETSAT antisense, 5'-AGA CGT AGC GCT CCA TCG CC-3'.

## Western blot analysis

Whole-cell protein extracts were obtained by direct lysis in Laemmli buffer heated to 100°C. Proteins resolved by SDS-PAGE were transferred to a polyvinylidene difluoride membrane (GE Healthcare) and probed with antibodies raised against 11 $\beta$ HSD1, 1:1000 (AB83522; Abcam); DHRS3, 1:1000 (15393-1-AP; ProteinTech Group); RETSAT, 1:1000 (SAB1407586; Sigma); and  $\beta$ -actin, 1:100 000 (A1978; Sigma). After incubation with horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 (DAKO), immunoreactivity was visualized using the ECL+ chemoluminescent detection kit (Amersham).

## Statistical analysis

Data were analyzed with the statistical package GraphPad Prism (GraphPad Software Inc). A Student's *t* test and a Mann-Whitney *U* test were used when appropriate. Logarithmic transformations were used when data were not normally distributed. Pearson's correlation coefficient (*r*) was used to assess the correlation between uNK cell densities in vivo and the induction of various genes upon decidualization of corresponding primary HESC cultures. Statistical significance was assumed when *P* < .05.

## Results

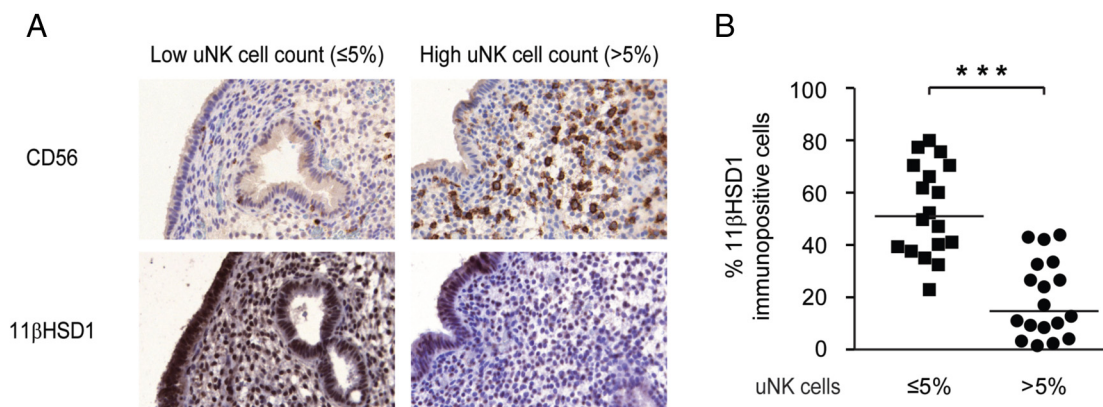
### Midluteal uNK cell density correlates inversely with endometrial 11 $\beta$ HSD1 expression

We routinely assess uNK cell densities by CD56 immunostaining of timed (d LH+7 to LH+10) endometrial biopsies from women suffering reproductive failure. Based on previous studies, normal uNK cell density is defined as 5% or less CD56+ cells in the stroma underlying the luminal epithelium (17, 23). We speculated that an apparent excess of uNK cells in the periimplantation endometrium may reflect impaired 11 $\beta$ HSD1 expression and relative cortisol deficiency. To test this hypothesis, a TMA was constructed using biopsies with normal (*n* = 18) as well as elevated (*n* = 18) uNK cell levels. The TMA was stained with anti-CD56 and anti-11 $\beta$ HSD1 antibodies. As shown in Figure 1A, a strong inverse correlation was observed between uNK cell density and 11 $\beta$ HSD1 immunoreactivity. This negative correlation was confirmed by semiquantitative image analysis (Figure 1B).

### Elevated uNK cell density in vivo is associated with impaired induction of key decidual markers in vitro

Previous studies have shown that an aberrant decidual response, associated with reproductive disorders such as





**Figure 1.** Inverse correlation between uNK cell density in the subluminal epithelium and expression of 11 $\beta$ HSD1. A, Representative CD56 and 11 $\beta$ HSD1 immunostaining in midluteal endometrial biopsies deemed to have normal or elevated uNK cell count ( $\leq 5\%$  or  $> 5\%$  CD56 $^{+}$  cells in the stroma, respectively). Original magnification,  $\times 20$ . B, Relative 11 $\beta$ HSD1 immunostaining after image analysis of a TMA containing biopsies with normal ( $\leq 5\%$ ;  $n = 18$ ) or increased ( $> 5\%$ ,  $n = 18$ ) uNK cell density in the subluminal stroma. \*\*\*,  $P < .001$ .

endometriosis and RPL, is maintained in culture (24, 25). This prompted us to investigate whether uNK cell density in vivo could reflect the induction of 11 $\beta$ HSD1 in response to decidual cues. To this end, we divided timed endometrial biopsies and processed one part for CD56 immunostaining and the other part for primary HESCs. These cultures were passaged once, grown to confluency, and treated with 8-bromo-cAMP, P4, and E for either 4 or 8 days. As shown in Figure 2, there was a striking inverse correlation between uNK cell density in vivo and the responsiveness of paired primary cultures to differentiation stimuli (Supplemental Table 1). This inverse correlation was apparent for the induction of not only *HSD11B1*, the gene that encodes 11 $\beta$ HSD1 (Figure 2A) but also for *PRL* and *IGFBP1*, two classical decidual marker genes (Figure 2, B and C).

We also examined whether this association extended to IL-11 and IL-15, cytokines implicated in regulating uNK cells (11–13). Rather surprisingly, there was a significant trend toward higher levels of induction IL-15, but not IL-11, transcripts in decidualizing HESC cultures obtained from biopsies with normal uNK cell densities (Figure 3, A and B).

### Elevated uNK cell density in vivo is associated with impaired expression of MR-dependent metabolic enzymes

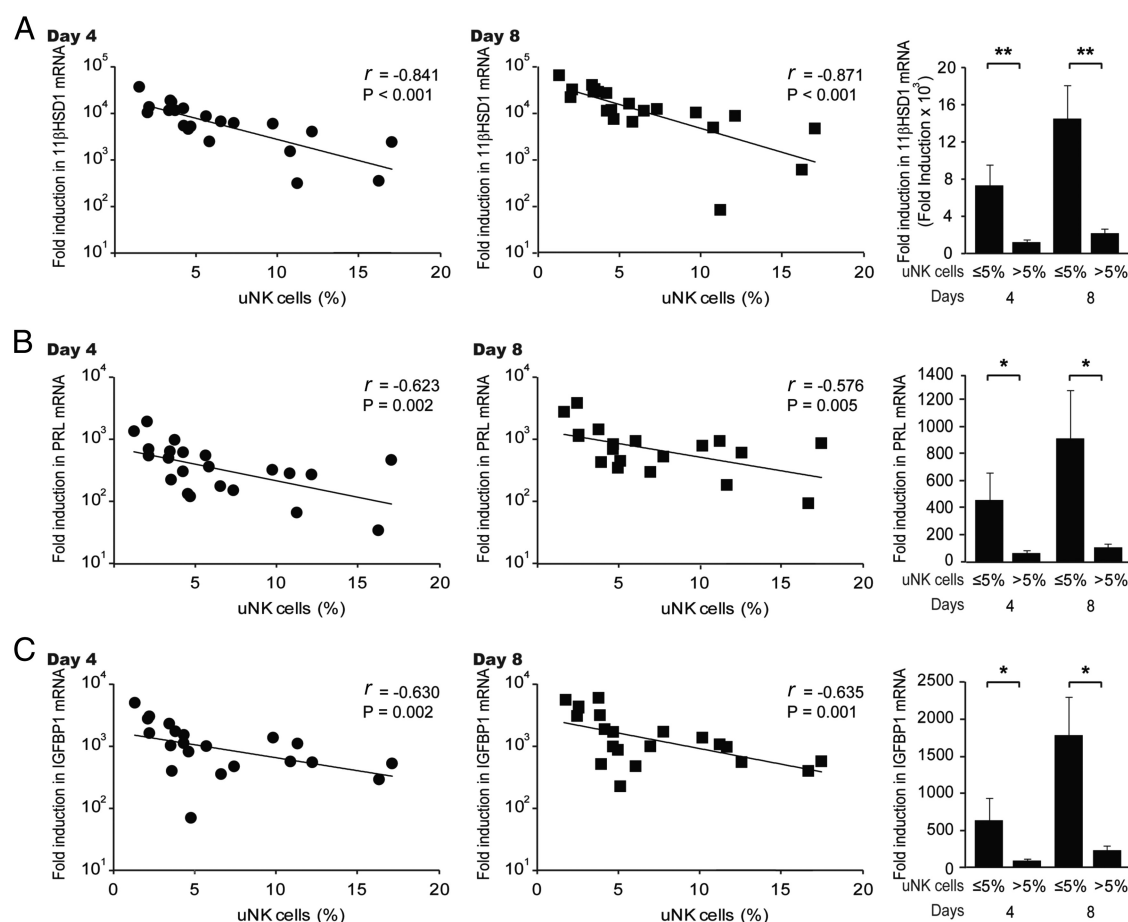
Next, we used the TMA to examine the expression of the cortisol-responsive receptors, GR and MR. As reported by others (26), GR and MR are both expressed in the endometrial stroma (Figure 4A). Semiquantitative analysis of the TMA showed no difference in stromal GR immunoreactivity in biopsies characterized by elevated vs normal uNK cell levels. In contrast, an increased percentage of CD56 $^{+}$  cell density was associated with significantly lower MR expression levels ( $P < .05$ ; Figure 4B).

Previous knockdown experiments in decidualizing HESCs have shown that the 11 $\beta$ HSD1/MR axis regulates the expression of several enzymes involved in lipid metabolism and retinoid acid biosynthesis and storage (18). We measured the expression levels of two target genes, *DHRS3* and *RETSAT*, to monitor the MR activity in decidualizing primary HESC cultures established from biopsies with normal or elevated uNK cell levels. Both genes were moderately induced upon treatment with 8-bromo-cAMP/P4/E in a time-dependent manner when the primary HESC cultures were established from samples with 5% or less CD56 $^{+}$  cells in the subepithelial stroma (Figure 5). By contrast, this induction was significantly impaired in cultures established from high uNK cell samples.

To validate these findings, we performed Western blot analysis of primary HESCs decidualized with 8-bromo-cAMP/P4/E for 4 days. As shown in Figure 6A, 11 $\beta$ HSD1 and DHRS3 were abundantly expressed in primary cultures decidualized for 4 days. Induction of RETSAT, however, requires prolonged decidualization. This enzyme was barely detectable after 4 days of differentiation and only in primary cultures established from samples with 5% or less CD56 $^{+}$  cells in the subepithelial stroma. Semiquantitative analysis of the blots showed that elevated uNK cell density in vivo is associated with significantly lower 11 $\beta$ HSD1 expression in corresponding decidualizing primary HESC cultures and a trend toward lower DHRS3 levels ( $P = .03$  and  $P = .07$ , respectively; Figure 6B).

### Discussion

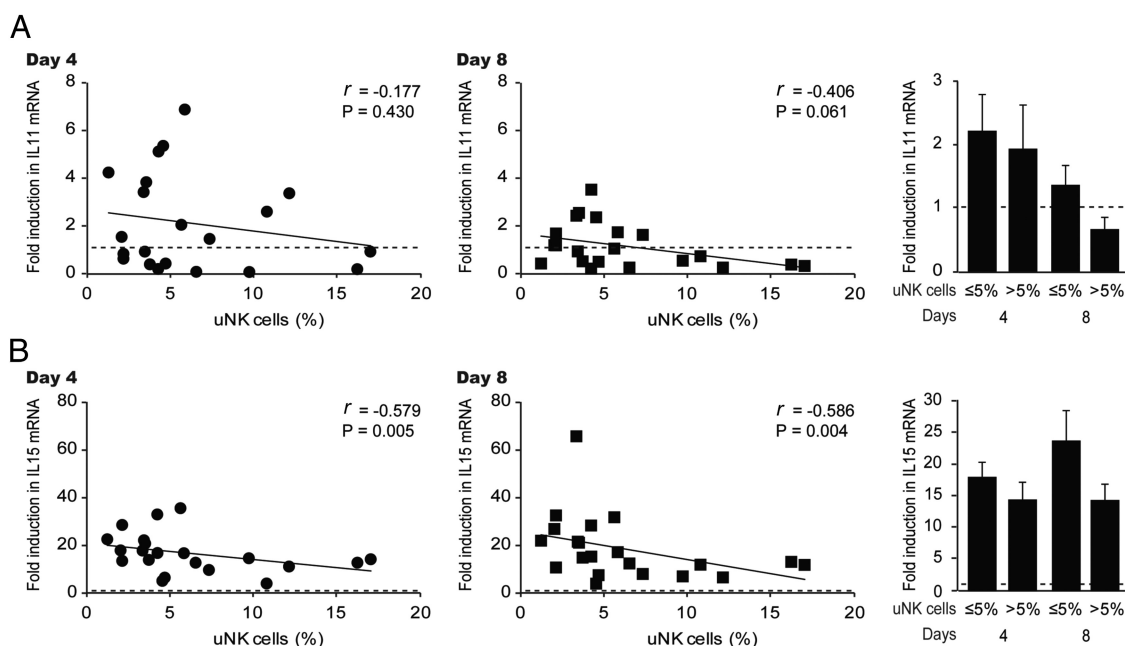
Increased uNK cell density in midluteal endometrium has been associated with reproductive failure, especially RPL (4–6). However, the mechanisms that account for cyclic



**Figure 2.** Inverse correlation between uNK cell densities in vivo and the induction of decidual markers in vitro. A, The uNK cell densities in midluteal biopsies correlated inversely to the induction of *11βHSD1* transcripts in primary HESCs decidualized for 4 days (left panel) or 8 days (middle panel). Note the logarithmic Y-axis. The right panel shows the mean ( $\pm$ SEM) induction of *11βHSD1* transcripts in biopsies deemed to have normal or elevated uNK cell counts. B, The uNK cell densities in midluteal biopsies correlated inversely to the induction of *PRL* transcripts in primary HESCs decidualized for 4 days (left panel) or 8 days (middle panel). The right panel shows the mean ( $\pm$ SEM) induction of *PRL* transcripts in biopsies deemed to have normal or elevated uNK cell counts. Note the logarithmic Y-axis. C, The uNK cell densities in midluteal biopsies correlated inversely to the induction of *IGFBP1* transcripts in primary HESCs decidualized for 4 days (left panel) or 8 days (middle panel). The right panel shows the mean ( $\pm$ SEM) induction of *PRL* transcripts in biopsies deemed to have normal or elevated uNK cell counts. Note the logarithmic Y-axis. \*,  $P < .05$ ; \*\*,  $P < .01$ .

recruitment of uNK cell precursors and subsequent proliferation and differentiation within the periimplantation environment are not well understood. In addition to IL-11 and IL-15, several other endometrial factors may be implicated in this process, including chemokine motif ligand 14, IL-12, and IL-33 (25, 27, 28). Different strands of evidence suggest that induction of a cortisol gradient upon decidualization of HESCs is also a key regulator of uNK cells in periimplantation endometrium. First, preconceptual prednisolone treatment markedly reduces uNK cell density in RPL patients (17). uNK cells express GR but not progesterone receptors (15), the inference being that glucocorticoids are likely to act directly on these cells. Second, we found a strong negative correlation between uNK cell densities and expression of *11βHSD1* in differentiating stromal cells in vivo. We have shown previously that *11βHSD1* expression and enzyme activity in decidualizing

HESCs is driven by cAMP and P4 signaling (18). Furthermore, inhibition of *11βHSD1* activity with either carbenoxolone disodium salt or PF 915275 virtually abolishes the induction of *HSD11B1*, indicating that local cortisol signaling reinforces the expression of this enzyme in decidualizing cells through an autocrine mechanism (18). By contrast, the expression of the type 2 isoform (*11βHSD2*), the dehydrogenase that converts cortisol into inactive cortisone, is low in both undifferentiated and decidualizing HESCs (18). Notably, the decidual process in the human endometrium is under tight spatiotemporal control (29). It is initiated in the midluteal phase of the cycle first in stromal cells surrounding the terminal spiral arteries and underlying the luminal epithelium. Thus, rather than the total tissue concentration of uNK cells, it is possible that excessive migration of uNK cells from their usual position in the basal and perivascular regions of the endometrium



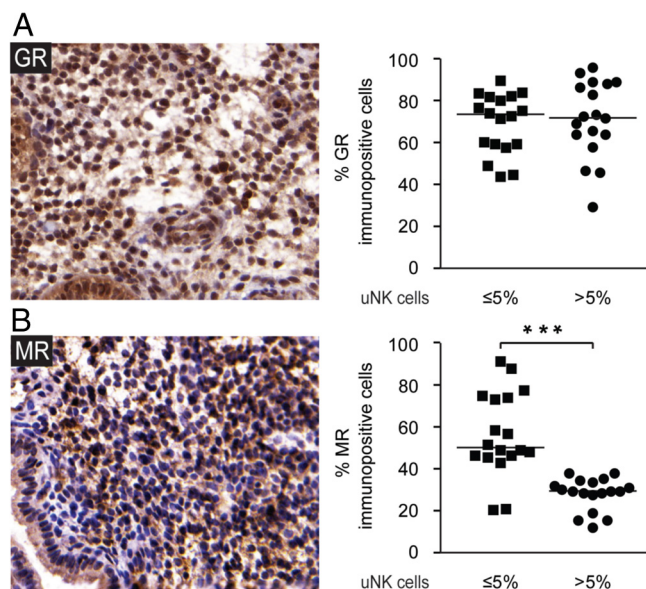
**Figure 3.** Induction of *IL11* and *IL15* mRNA in decidualizing HESCs in culture and uNK cell densities in vivo. A and B, The uNK cell densities in midluteal biopsies were correlated to the induction of *IL11* and *IL15* transcripts, respectively, in corresponding primary HESCs decidualized for either 4 days (left panel) or 8 days (middle panel). The right panel shows the mean ( $\pm$ SEM) induction in biopsies deemed to have normal or elevated uNK cell counts.

to the subluminal region is the hallmark of an abnormal decidual response that predisposes for early pregnancy loss. Finally, there is increasing evidence that the responsiveness of endometrial cells to differentiation signals is subject to epigenetic programming (30), which explains

how an aberrant decidual response in vivo is maintained, at least partly, upon differentiation of purified HESCs in vitro (24, 25, 31). In agreement, we found that high uNK cell density in vivo is associated with blunted induction of *HSD11B1* as well as decidual marker genes, such as *PRL* and *IGFBP1*, in primary cultures. This strong inverse correlation suggests that the 5% threshold of uNK cell density is somewhat arbitrary. Whether increased uNK cells densities correlate to increased risk of pregnancy failure warrants further investigation.

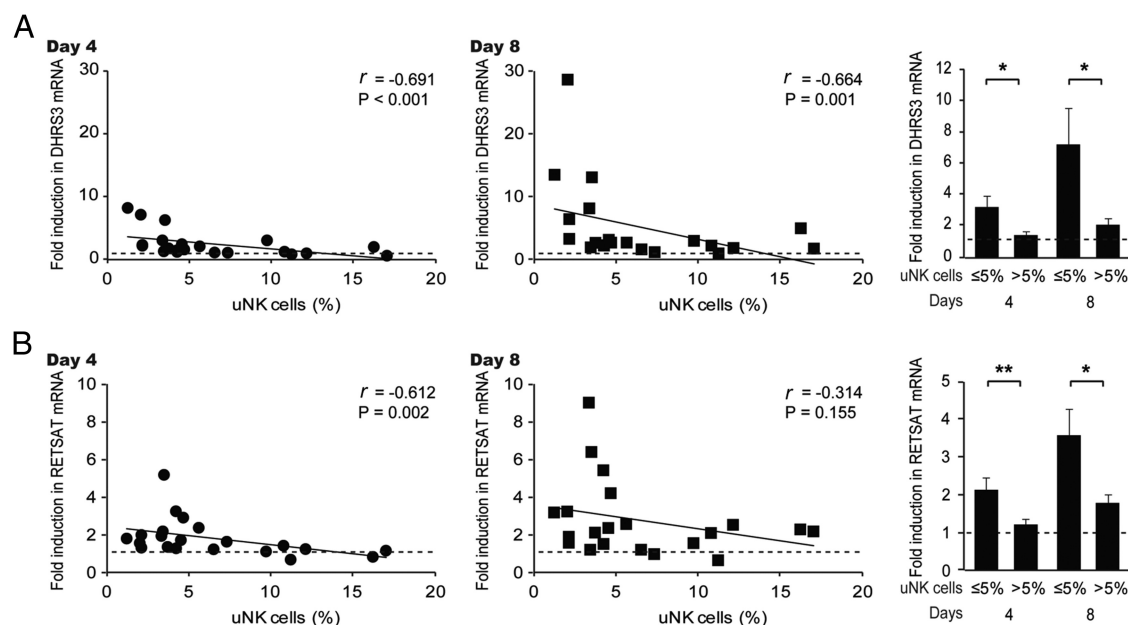
In addition to decidual marker genes, induction of IL-11 and IL-15 also tended to be lower in decidualizing cells established from biopsies with elevated uNK cell levels. This observation does not exclude that expression levels of these cytokines in situ correlate with uNK cell levels as reported for IL-15 in a recent study (32).

Human uNK cells have been described as immature and inactive before pregnancy (1). How elevated levels of uNK cells prior to conception predispose to subsequent pregnancy failure is unclear. Ablation of these cells in mice has been shown to compromise spiral arteriole remodeling and maintenance of decidual integrity seen after midpregnancy (3, 33). Yet uNK cell-deficient *IL-15*<sup>-/-</sup> mice are fertile and have normal gestation lengths and litter sizes comparable with wild-type mice (34). Similarly, human uNK cells are implicated in spiral artery remodeling. They are a rich source of angiogenic growth factors, although paradoxically the endometrium of RPL patients is characterized by reduced expression of several key factors, in-



**Figure 4.** Increased uNK cell levels are associated with impaired MR expression. A and B, The expression of GR and MR, respectively, in the subluminal stroma was assessed by immunostaining of a TMA containing biopsies with normal ( $\leq 5\%$ ;  $n = 18$ ) or increased ( $> 5\%$ ,  $n = 18$ ) uNK cell density in the subluminal stroma. The left panel shows representative immunostaining (original magnification,  $\times 40$ ), whereas the right panel depicts semiquantitative image analysis of the immune staining. \*\*\*,  $P < .001$ .



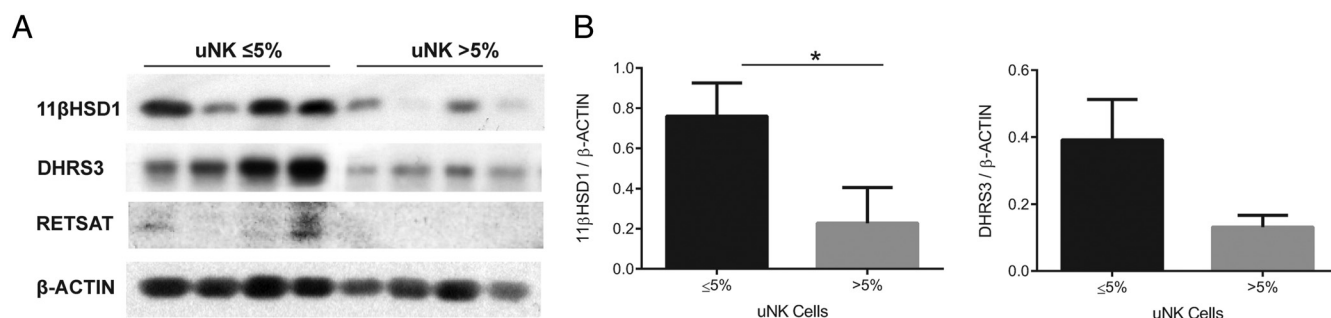


**Figure 5.** Elevated uNK cell density is associated with blunted expression of the MR-dependent genes in decidualizing HESCs. A, The uNK cell densities in 21 biopsies correlated inversely to the induction of *DHRS3* transcripts in primary HESCs decidualized for 4 days (left panel) or 8 days (middle panel). The right panel shows the mean ( $\pm$ SEM) induction of *DHRS3* transcripts in biopsies deemed to have normal or elevated uNK cell counts. B, The uNK cell densities in 21 biopsies correlated inversely to the induction of *RETSAT* transcripts in primary HESCs decidualized for 4 days (left panel) or 8 days (middle panel). The right panel shows the mean ( $\pm$ SEM) induction of *RETSAT* transcripts in biopsies deemed to have normal or elevated uNK cell counts. \*,  $P < .05$ ; \*\*,  $P < .01$ .

cluding platelet-derived growth factor-BB, angiotensin-2, vascular endothelial growth factor-A, and vascular endothelial growth factor-C (35). Our data suggest that increased density of CD56<sup>+</sup> cells in the subluminal endometrial stromal compartment may be an indirect marker of local corticosteroid deficiency. Furthermore, our data indicate that the 11 $\beta$ HSD1/MR axis in target cells may be particularly affected as exemplified by the impaired induction of *DHRS3* and *RETSAT* transcripts in decidualizing cultures established from high uNK cell biopsies. These enzymes are involved in lipid metabolism and retinoid acid (RA) biosynthesis and storage. Both shortage and excess of RA contribute to fetal malformation, suggesting that retinoid metabolism must be regulated closely at the fetomaternal interface (36). Alcohol dehydrogenase

and nicotinamide adenine dinucleotide phosphate oxidase-dependent short-chain dehydrogenases/reductases, including *DHRS3*, oxidize retinal to retinol and promote its storage as retinyl esters. Likewise, *RETSAT* is involved in the regulation of retinoid storage as lipid droplets (37). Interestingly, RA inhibits decidualization of HESCs, and excess levels of RA or retinal are cytotoxic (38). Thus, high uNK cell density in the periimplantation endometrium may be associated with perturbations in the retinoid metabolism pathway in the stromal compartment, which in turn predisposes for an impaired decidual response and compromise histiotrophic support of the early conceptus.

Thus, it seems likely that complex and dynamic gradients of chemoattractants and chemorepellents control the spatiotemporal distribution of uNK cells in the periim-



**Figure 6.** A, Composite figure showing 11 $\beta$ HSD1, *DHRS3*, and *RETSAT* protein expression in primary HESC cultures decidualized for 4 days. A total of eight primary cultures were established from biopsies with normal or elevated uNK cell densities.  $\beta$ -Actin served as a loading control. B, Semiquantitative analysis of 11 $\beta$ HSD1 and *DHRS3* expression relative to  $\beta$ -actin. Because of the low level of expression, *RETSAT* expression was not quantified. \*,  $P < .05$ .

plantation endometrium. In addition to glands and other immune cells, decidualizing stromal cells play a major role in governing this process, at least partially, by inducing a cortisol gradient that establishes a nutritive environment essential for postimplantation embryo development and fetal growth. Our data suggest that excessive uNK cells in the subluminal stromal compartment prior to conception may serve as a potential biomarker for a suboptimal decidual response in pregnancy. A recent pilot randomized, double-blind controlled clinical trial suggested an improvement in live birth rate with prednisolone in women with RPL and high midluteal uNK cell density (23), although this finding needs validating in a larger trial. In addition, assessment of uNK cell density varies greatly from laboratory to laboratory and intercycle variation has been reported (22). To be clinically useful, international standardization of uNK cell assessment is urgently needed.

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# Induction of 11 $\beta$ -HSD 1 and Activation of Distinct Mineralocorticoid Receptor- and Glucocorticoid Receptor-Dependent Gene Networks in Decidualizing Human Endometrial Stromal Cells

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The actions of glucocorticoids at the feto-maternal interface are not well understood. Here, we show that decidualization of human endometrial stromal cells (HESCs) in response to progesterone and cAMP signaling is associated with a strong induction of 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1) expression and enzyme activity. Decidualization also triggered a gradual decrease in glucocorticoid receptor (GR) expression and reciprocal increase in mineralocorticoid receptor (MR) levels. Gene expression profiling of differentiating HESCs after small interfering RNA (siRNA)-mediated knockdown of either GR or MR identified 239 and 167 significantly regulated genes, respectively. Interestingly, GR-repressed genes were enriched for Krüppel-associated box domain containing zinc-finger proteins, transcriptional repressors involved in heterochromatin formation. In agreement, GR knockdown was sufficient to enhance trimethylated H3K9 levels in decidualizing cells. Conversely, we identified several MR-dependent genes implicated in lipid droplet biogenesis and retinoid metabolism. For example, the induction in differentiating HESCs of *DHRS3*, encoding a highly conserved enzyme that catalyzes the oxidation/reduction of retinoids and steroids, was enhanced by aldosterone, attenuated in response to MR knockdown, and abolished upon treatment with the MR antagonist RU26752. Furthermore, we demonstrate that decidualization is associated with dynamic changes in the abundance and distribution of cytoplasmic lipid droplets, the formation of which was blocked by RU26752. In summary, progesterone drives local cortisol biosynthesis by decidual cells through induction of 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ HSD1), leading to transcriptional regulation of distinct GR and MR gene networks involved in epigenetic programming and lipid and retinoid metabolism, respectively. (*Molecular Endocrinology* 27: 192–202, 2013)

**G**lucocorticoids have been implicated in many processes that underpin successful embryo implantation, placentation, and the growth and development of the fetus (1). Consequently, modulation of glucocorticoid action represents a potential strategy for the treatment or prevention of a variety of pregnancy-related disorders.

For example, glucocorticoid treatment has been advocated for the prevention of early pregnancy loss based on its ability to reduce the abundance of uterine natural killer cells during the periimplantation window (2), to stimulate human chorionic gonadotropin secretion by cultured human cytotrophoblasts (3), and to accelerate trophoblast

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Abbreviations: AR, Androgen receptor; 8-bromo-cAMP, 8-bromoadenosine cAMP; CBX, carbenoxolone disodium salt; DCC-FBS, dextran-coated charcoal-treated fetal bovine serum; DEX, dexamethasone; DHRS, dehydrogenase/reductase superfamily; E, cortisone; ER, endoplasmic reticulum; F, cortisol; FBXO32, F-box only protein 32; GR, glucocorticoid



growth and invasion (4). However, implantation is an inflammatory process that depends on local release of proinflammatory cytokines and prostaglandins (5–8). By disabling the cytokine-prostaglandin signaling cascade, glucocorticoids potentially could impact adversely on early pregnancy events. Furthermore, compelling evidence suggests that prolonged exposure to high levels of glucocorticoids in pregnancy is detrimental for both placental and fetal development (9–11).

Tissue levels of active endogenous glucocorticoids depend on the expression of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ HSD) enzymes. Although the 11 $\beta$ HSD1 isoform is a bidirectional enzyme, it predominantly catalyzes the conversion of inert cortisone (E) to active cortisol (F), thus increasing tissue levels of active glucocorticoids (12). The reduced nicotinamide adenine dinucleotide [NAD(H)]-dependent type 2 isoform (11 $\beta$ HSD2) acts as a dehydrogenase, converting F to E (13). The main cellular targets for F are glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). However, GR normally colocalizes with 11 $\beta$ HSD1 *in vivo*, whereas 11 $\beta$ HSD2 is commonly found in MR-expressing tissues. Nevertheless, hypercortisolism can result in apparent mineralocorticoid excess as in Cushing syndrome (14). Further, some cell types like adipocytes do not significantly express 11 $\beta$ HSD2, thus enabling F to act through MR (15, 16).

Both 11 $\beta$ HSD isoforms as well as GR and MR are expressed in human endometrium (17). Interestingly, GR expression is confined to stromal cells, whereas MR is reportedly present in both stromal and glandular compartments. Further, 11 $\beta$ HSD1, but not 11 $\beta$ HSD2, is highly up-regulated upon differentiation of primary human endometrial stromal cells (HESCs) into specialist decidual cells *in vitro* (18). This progesterone (P4)-driven differentiation process bestows unique functions on the endometrium that are essential for pregnancy, including the ability to regulate trophoblast invasion, to modulate local angiogenesis, to recruit specialized uterine natural killer cells and macrophages, and to resist environmental and oxidative stress (19, 20). Based on these observations, we speculated that induction of 11 $\beta$ HSD1 would lead to increased F bioavailability, which in turn regulates the expression of distinct GR- and MR-dependent gene networks in decidualizing HESCs.

receptor; GRIA1, glutamate receptor, ionotropic, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid; HESC, human endometrial stromal cell; H3K9, histone 3 lysine 9; H3K9me3, H3K9 trimethylation; 11 $\beta$ HSD, 11 $\beta$ -hydroxysteroid dehydrogenase; IGFBP1, IGF-binding protein 1; KRAB-ZNF, Krüppel-associated box domain containing ZNF; MPA, medroxyprogesterone acetate; MR, mineralocorticoid receptor; NT, nontargeting; P4, progesterone; PF, PF 915275; PR, P4 receptor; PRL, prolactin; RA, retinoic acid; RTQ, real-time quantitative; siRNA, small interfering RNA; WINT4, wingless-type MMTV integration site family, member 4; ZNF, zinc-finger.

## Materials and Methods

### Patient selection

This study was approved by the Hammersmith and Queen Charlotte's and Chelsea Research Ethics Committee (1997/5065). Written informed consent was obtained from all participating subjects before tissue collection. Endometrial biopsies were timed between 7 and 11 d after the preovulatory LH surge.

### Primary cell culture

Endometrial samples were obtained from premenopausal women without uterine pathology, and HESCs were isolated, cultured, and maintained as described previously (16). Samples were collected in Earle's buffered saline containing 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. The tissues were washed twice in a 1:1 mixture of DMEM and Ham's F12 (DMEM/F12) (Sigma, Poole, UK), finely minced, and enzymatically digested with collagenase (134 U/ml) and deoxyribonuclease type I (156 U/ml) (Sigma) for 1 h at 37 C. After centrifugation at 400  $\times$  g for 4 min, the pellet was resuspended in maintenance medium of DMEM/F12 containing 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS), 1% L-glutamine, and 1% antibiotic-antimycotic solution. Proliferating HESCs were cultured in maintenance medium until confluence. In decidualization experiments, confluent monolayers were maintained in DMEM/F12 now containing 2% DCC-FBS and treated with 0.5 mM 8-bromoadenosine cAMP (8-bromo-cAMP) (Sigma, St. Louis, MO) alone or in combination with 1  $\mu$ M medroxyprogesterone acetate (MPA) (Sigma), 1  $\mu$ M P4 (Sigma), 0.1  $\mu$ M dexamethasone (DEX) (Sigma), 0.1  $\mu$ M E (Sigma), 1  $\mu$ M aldosterone (Sigma), 10  $\mu$ M RU26752 (Sigma), 0.1  $\mu$ M carbenoxolone disodium salt (CBX) (Sigma), or 0.1  $\mu$ M PF 915275 (PF) (Tocris Bioscience, Abingdon, UK).

### Transient transfection

Primary HESCs, cultured in 12-well plates until confluency, were transfected using the ProFection mammalian transfection kit (Promega, Madison, WI), with 100 nM per well of the following small interfering RNA (siRNA) reagents (Dharmacon, Lafayette, CO): siCONTROL nontargeting (NT) siRNA pool, GR siGENOME SMARTpool siRNA, or MR siGENOME SMARTpool siRNA.

### Western blot analysis

Whole-cell protein extracts were obtained by direct lysis in Laemmli buffer heated to 100 C. Proteins resolved by SDS-PAGE were transferred to a polyvinylidene difluoride membrane (GE Healthcare, Uppsala, Sweden) and probed with antibodies raised against GR, 1:1000 (E-20: SC-1003; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); MR, 1:1000 (H-300: SC-11412; Santa Cruz Biotechnology, Inc.); 11 $\beta$ HSD1, 1:1000 (ab83522; Abcam, Cambridge, UK); 11 $\beta$ HSD2, 1:2000 (ab80317; Abcam); P4 receptor (PR), 1:1000 (NCL-L-PGR-312; Leica Biosystems, Newcastle, UK); histone 3 lysine 9 (H3K9) trimethylation (H3K9me3), 1:1000 (49-1008; Invitrogen Ltd., Paisley, UK); dehydrogenase/reductase superfamily (DHRS)3, 1:1000 (15393-1-AP; Proteintech Group, Manchester, UK); and  $\beta$ -actin, 1:100,000 (A1978; Sigma). After incubation with horseradish peroxidase-conjugated secondary antibodies, 1:2500, (Roche Diagnostics, Mannheim, Germany), chemiluminescence

was visualized using the ECL+ chemiluminescence detection kit (Amersham, Little Chalfont, UK).

### Real-time quantitative (RTQ)-PCR

Total RNA was extracted from primary HESC cultures. After treatment with amplification grade deoxyribonuclease I (Invitrogen Ltd.), cDNA was generated using the SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen Ltd.). Template quantification was performed with an ABI Step One System (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems) as dye layer and the relative standard curve calculation method. RNA input variances were normalized against the levels of the L19 house-keeping gene, which encodes a ribosomal protein. All measurements were performed in duplicate. Specific primer pairs were designed using Primer3 software (<http://frodo.wi.mit.edu>): L19 sense, 5'-GCG GAA GGG TAC AGC CAA T-3' and L19-R antisense, 5'-GCA GCC GGC GCA AA-3'; 11 $\beta$ HSD1 sense, 5'-AGC AAG TTT GCT TTG GAT GG-3' and 11 $\beta$ HSD1 antisense, 5'-AGA GCT CCC CCT TTG ATG AT-3'; GR sense, 5'-CCC TAC CCT GGT GTC ACT GT-3' and GR antisense, 5'-GGT CAT TTG GTC ATC CAG GT-3'; MR sense, 5'-GGC ACT CGC TGG CCT GGA TG-3' and MR antisense, 5'-GTC TCC ATC GCT GCC TCG GC-3'; decidual prolactin (PRL) sense, 5'-AAG CTG TAG AGA TTG AGG AGC AAA C-3' and decidual PRL antisense, 5'-TCA GGA TGA ACC TGG CTG ACT A-3'; IGF-binding protein 1 (IGFBP1) sense, 5'-CGA AGG CTC TCC ATG TCA CCA-3' and IGFBP1 antisense, 5'-TGT CTC CTG TGC CTT GGC TAA AC-3'; wingless-type MMTV integration site family, member 4 (WNT4) sense, 5'-TCA GCC CAC AGG GCT TCC AGT-3' and WNT4 antisense, 5'-CGG CTC GCC AGC ACG TCT TT-3'; F-box only protein 32 (FBXO32) sense, 5'-GCG GCA GTT TCG TGA GCG AC-3' and FBXO32 antisense, 5'-GGG TGC AAT ATC CAT GGC GCT CTT-3'; glutamate receptor, ionotropic, 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid (GRIA1) sense, 5'-AAG GGG TCT GCC CTG AGA AAT CCA-3' and GRIA1 antisense, 5'-ACG CCT GCC ACA TTG CTG AGG-3'; zinc-finger (ZNF)486 sense, 5'-CTG GAG GAG TGG CAT TGC CTG G-3' and ZNF486 antisense, 5'-ACA CAC AAC TGG GGG TTT GGC AAT-3'; and DHRS3 sense, 5'-AGC GCG GCG CCA GAA AGA TT-3' and DHRS3 antisense, 5'-TCA CCC ACC TTC TCC CGG ACG-3'.

### Radio-thin layer chromatography

The activity of 11 $\beta$ HSD1 in HESCs was analyzed essentially as described in literature (21, 22). Briefly, the microsome-containing fraction of undifferentiated and decidualized HESCs was incubated with reduced nicotinamide adenine dinucleotide phosphate (NADPH) (1 mM) and [ $^3$ H] E. [ $^{14}$ C] F ( $3 \times 10^3$  dpm) was added to monitor procedural losses together with 50  $\mu$ g of unlabeled F. The assay was performed under initial rate conditions, and less than 12% of the substrate was converted to product. Precursor and product steroids were separated by thin layer chromatography using chloroform: methanol (9:1, vol/vol). Retention factor values for F and E were 0.5 and 0.8, respectively. The amount of radioactivity was measured by scintillation spectrometry. Enzyme activity was determined as the amount of product formed per milligram of protein per hour after correction for procedural losses.

### Microarray analysis

Genome-wide microarray analysis was performed on primary cultures established from four different patients. Each culture was first transfected either with NT-siRNA or siRNA oligos against MR or GR. The cells were then decidualized for 4 d with 8-bromo-cAMP, P4, and E. Total RNA was extracted using STAT-60 reagent (AMS Biotechnology, Abingdon, UK). RTQ-PCR was performed after first-strand cDNA synthesis to determine the level of GR and MR knockdown. RNA quality was analyzed on an Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany). Microarray analysis on total RNA samples was performed by UCL Genomics using Bioconductor version 2.0 and R version 2.9.0. The robust multiarray analysis algorithm was used to obtain normalized data and gene signals. This method performs within-chip and between-chip normalizations in a single step. Gene summaries are generated using the Affymetrix Expression Console software. The criteria used to generate lists of differentially expressed genes are based on standard filtering by fold change ( $\pm 50\%$  change) and include false discovery rate filter ( $P \leq 0.05$ ). Gene ontology annotation was performed using the Database for Annotation, Visualization, and Integrated Discovery Bioinformatics Resources 6.7 (SAIC-Frederick, Inc., Frederick, MD). Microarray data have been deposited in GEO, accession number GSE42538.

### Confocal microscopy and lipid droplets staining

Primary HESCs cultured on glass slides were fixed with 4% paraformaldehyde (Sigma) and permeabilized by 0.1% Triton X-100 (BDH Chemicals, London, UK). BODIPY 493/503 (Invitrogen Ltd.) was applied at 1  $\mu$ g/ml in PBS. BODIPY-stained samples had to be washed with PBS before imaging. 4,6-Diamidino-2-phenylindole was used to identify nuclei. We examined samples under epifluorescent optics, and digital images were obtained with a Zeiss 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

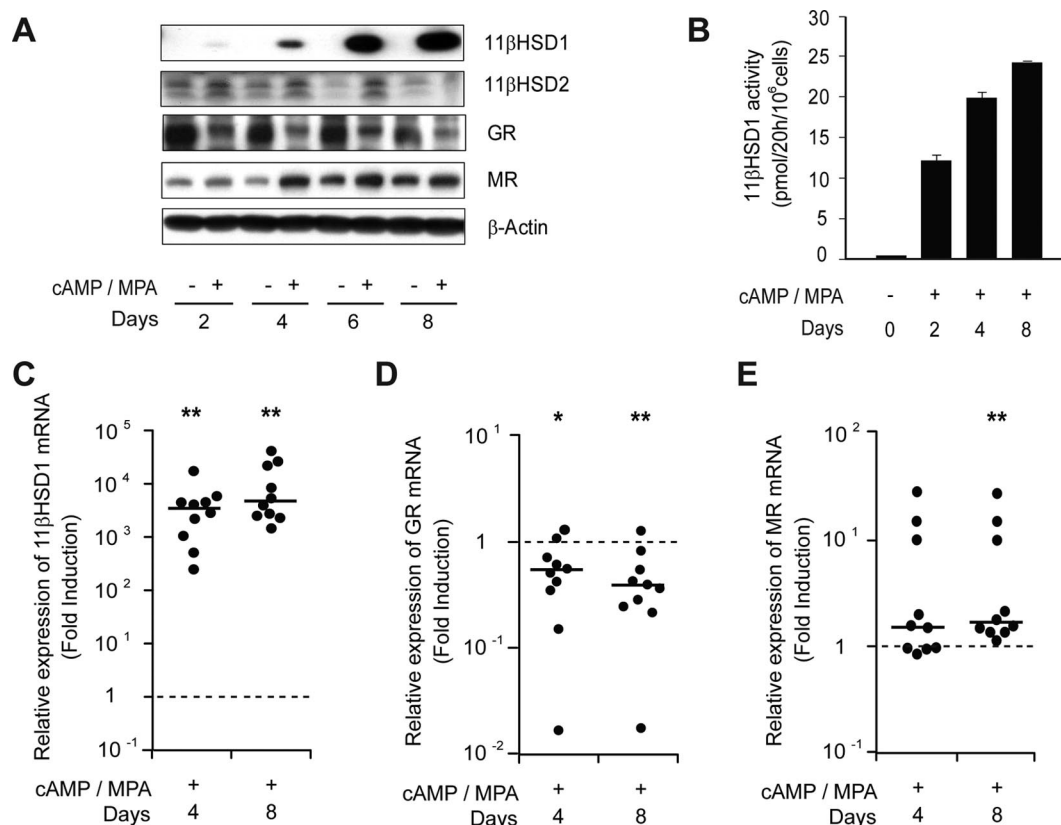
### Statistical analysis

Data presented in this study are representative of four or more biological replicates (*i.e.* primary cultures established from different biopsies). Statistical analysis was performed using a Student's *t* test after normalization of the data. The level of significance was defined as  $P < 0.05$ .

## Results

### Expression of 11 $\beta$ HSDs, GR, and MR in undifferentiated and decidualized HESCs

To examine whether corticosteroid signaling plays a role in decidualization, we first examined the expression levels of 11 $\beta$ HSD1, 11 $\beta$ HSD2, GR, and MR in undifferentiated HESCs and cells differentiated with 8-bromo-cAMP and MPA for 2, 4, 6, or 8 d. As shown in Fig. 1A, decidualization of HESCs was associated with a remarkable induction of 11 $\beta$ HSD1 in a time-dependent manner. This was paralleled by a strong increase in 11 $\beta$ HSD1 enzyme activity in decidualizing cells (Fig. 1B). In con-



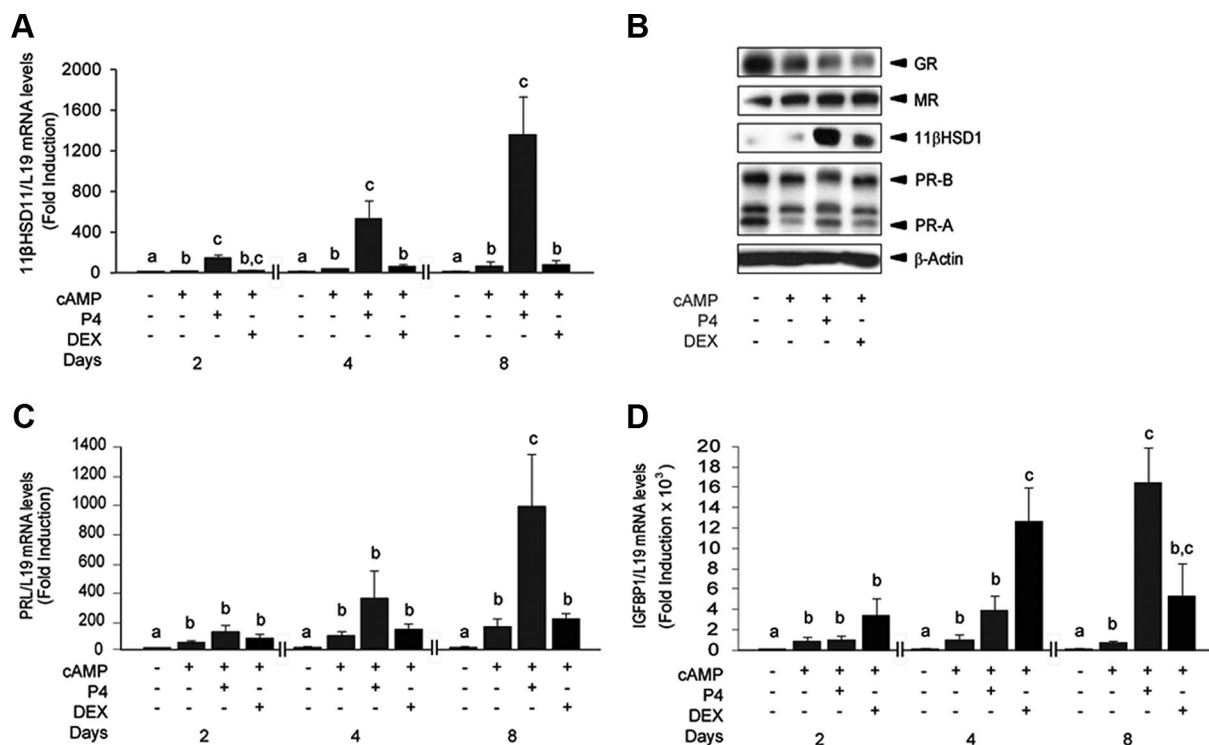
**FIG. 1.** Expression of 11 $\beta$ HSD enzymes, GR, and MR in decidualizing HESCs. **A**, Primary HESCs were treated with 8-bromo-cAMP and MPA in time-course experiments lasting 8 d. Total protein lysates were harvested at the indicated time points and subjected to Western blot analysis for 11 $\beta$ HSD1, 11 $\beta$ HSD2, GR, and MR.  $\beta$ -Actin served as a loading control. **B**, 11 $\beta$ HSD1 activity was measured using radio-thin layer chromatography in HESCs treated with 8-bromo-cAMP and MPA for the indicated time points. **C**, Induction of 11 $\beta$ HSD1 mRNA in 10 independent primary cultures treated with 8-bromo-cAMP and MPA for either 4 or 8 d. The data show fold change in expression relative to levels in undifferentiated HESCs (dotted line). Note the logarithmic y-axis. The horizontal bar indicates median. \*,  $P < 0.05$ ; \*\*,  $P < 0.001$ . **D** and **E**, The same sample set was analyzed for GR and MR, respectively.

trast, 11 $\beta$ HSD2 expression was low in HESCs, and in the absence of treatment with 8-bromo-cAMP and MPA, expression further declined in response to low-serum (2% DCC-FBS) culture conditions. Undifferentiated HESCs also abundantly express GR. However, treatment with 8-bromo-cAMP and MPA down-regulated this nuclear receptor (Fig. 1A). MR followed a reversed pattern with expression gradually rising in differentiating HESCs. Notably, culturing HESCs in low-serum conditions for several days was sufficient to alter MR and GR expression in opposite directions, although the effect was much more pronounced upon 8-bromo-cAMP and MPA treatment (Fig. 1A).

We next examined the regulation of 11 $\beta$ HSD1 mRNA in response to differentiation signals in 10 independent primary cultures. As shown in Fig. 1C, 11 $\beta$ HSD1 transcript levels invariably increased by several orders of magnitude in response to 8-bromo-cAMP and MPA treatment. Furthermore, the down-regulation of GR and reciprocal induction of MR at protein level in decidualizing cells were reflected at transcript level (Fig. 1, D and E).

#### P4 drives the expression of 11 $\beta$ HSD1 in decidualizing HESCs

MPA is a progestin with significant glucocorticoid actions. We therefore used P4 and DEX to examine whether either PR or GR drives the induction of 11 $\beta$ HSD1 in differentiating HESCs. Time-course analysis demonstrated that, in contrast to P4, DEX had little or no effect on the induction of 11 $\beta$ HSD1 transcripts in 8-bromo-cAMP-treated cells (Fig. 2A). Compared with treatment with 8-bromo-cAMP alone, addition of DEX did up-regulate 11 $\beta$ HSD1 protein levels, although this response was much more pronounced with 8-bromo-cAMP and P4 (Fig. 2B). Western blot analysis also revealed that cAMP signaling drives the down-regulation of GR and reciprocal induction of MR in decidualizing cells (Fig. 2B). As previously reported (23), 8-bromo-cAMP reduces cellular levels of PR isoforms (PR-A and PR-B). Although cotreatment with P4 induces a mobility shift in PR migration on SDS-PAGE, reflecting ligand-dependent receptor phosphorylation, DEX had no effect (Fig. 2B). We also com-



**FIG. 2.** Glucocorticoids selectively modulate the expression of decidual marker genes. A, RTQ-PCR analysis of 11βHSD1 transcript levels in HESCs treated with 8-bromo-cAMP with or without P4 or DEX for 2, 4, and 8 d. The results show the fold change (mean ± SEM) in 11βHSD1 transcript levels relative to vehicle control of four independent primary cultures. B, Western blot analysis of GR, MR, 11βHSD1, and PR proteins in whole-cell lysates obtained from primary HESCs treated with a combination of 8-bromo-cAMP with or without P4 or DEX for 4 d. β-Actin served as a loading control. C and D, Fold change (mean ± SEM) in PRL and IGFBP1 mRNA levels, respectively, relative to vehicle control of four independent primary cultures. Different letters above the error bars indicate that those groups are significantly different from each other at  $P < 0.05$ .

pared the induction of two decidual markers, PRL and IGFBP1, in primary cultures with 8-bromo-cAMP and either P4 or DEX. Unlike P4, DEX had no effect on cAMP-dependent induction of PRL transcripts in HESCs (Fig. 2C). However, DEX was more potent than P4 in enhancing IGFBP1 expression in cultures stimulated with 8-bromo-cAMP, especially during the early stages of the decidual process (Fig. 2D).

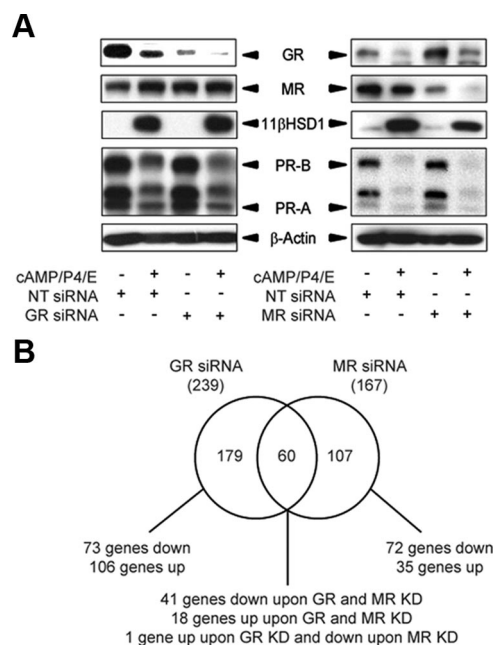
To test whether 11βHSD1-dependent F biosynthesis modulates the expression of decidual marker genes, we decidualized primary HESCs with 8-bromo-cAMP and P4 in the presence or absence of E. Addition of E has no effect on the induction of PRL. However, it strongly enhanced the expression of IGFBP1 and, albeit less pronounced, 11βHSD1 transcripts (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>). Conversely, treatment of primary cultures with an 11βHSD1 inhibitor (CBX or PF) attenuated the induction of IGFBP1 and HSD11B1 but not PRL (Supplemental Fig. 2). These observations underscore that 11βHSD1-dependent F biosynthesis impacts selectively on the expression of decidual marker genes.

### Identification of GR- and MR-dependent genes in decidualizing HESCs

Next, we set out to identify GR- and MR-dependent genes responsive to endogenous F biosynthesis in differentiating HESCs. To do this, four individual primary cultures were first transfected with NT-, GR-, or MR-siRNA and then treated with 8-bromo-cAMP, P4, and E for 4 d. Total mRNA and protein lysates were extracted from parallel cultures. Western blot analysis was used to confirm GR and MR knockdown (Fig. 3A). GR knockdown had no impact on MR, 11βHSD1, or PR levels. In contrast, MR silencing seemed to hinder the induction of 11βHSD1 and modestly up-regulated GR levels.

Total RNA extracted from decidualizing cultures transfected with NT-, GR-, or MR-siRNA was processed for whole genome microarray analysis. Using a cut-off of more than or equal to 1.5-fold change, 179 and 107 GR- and MR-selective genes, respectively, were identified ( $P < 0.05$ ) (Fig. 3B). Interestingly, GR knockdown resulted in significantly more up- than down-regulated genes (106 vs. 73, respectively;  $\chi^2$  test,  $P < 0.001$ ), whereas MR knockdown had the opposite effect (up- and down-regulated genes: 35 vs. 72, respectively;  $\chi^2$  test,  $P < 0.001$ ). Supplemental Tables 1–4 list the genes induced or re-





**FIG. 3.** Identification of GR- and MR-regulated genes in decidualizing HESCs. A, Western blot analysis of GR, MR, 11 $\beta$ HSD1, and PR expression in protein lysates extracted from primary cultures first transfected with NT-, GR-, or MR-siRNA and then treated with 8-bromo-cAMP, P4, and E for 4 d.  $\beta$ -Actin served as a loading control. C, Venn diagram showing the number of differentially expressed genes in decidualizing cells treated with 8-bromo-cAMP, P4, and E for 4 d in response to GR or MR knockdown.

pressed by GR and MR. We also identified 60 genes under control of both nuclear receptors in decidualizing HESCs with only a single gene, *GRIA1*, regulated in an opposing manner (Supplemental Table 5). We chose *GRIA1* as well as *WNT4* and *FBXO32* (putative GR- and MR-dependent genes, respectively) for initial validation of the array findings. Using independent cultures, we first monitored the expression of these genes in response to treatment with 8-bromo-cAMP, P4, and E for 4 or 8 d and then examined the impact of either GR or MR knockdown. *GRIA1*, which encodes for one of the four ionotropic 2-amino-3-(3-hydroxyl-5-methyl-isoxazol-4-yl)propanoic acid receptor subunits (24), is profoundly repressed in differentiating cells with transcript levels declining more than 90% after 4 d of treatment (Fig. 4A). This repression was partially relieved upon GR knockdown but enhanced in response to MR silencing (Fig. 4B). *WNT4* and *FBXO32* are both induced upon decidualization predominantly in a GR- and MR-dependent manner, respectively (Fig. 4). Interestingly, *FBXO32*, which encodes the E3 ubiquitin ligases Atrogin-1, was recently shown to be an 11 $\beta$ HSD1-dependent gene in skeletal muscle cells (25). In agreement, treatment of primary cultures with either CBX or PF inhibited *FBXO32* and *GRIA1* expression in decidualizing cells *PRL* (Supplemental Fig. 2). In case of *GRIA1*, this suggests that MR-dependent induction may be dominant over GR-depen-

dent inhibition. However, 11 $\beta$ HSD1 inhibition had no significant effect on the induction of *WNT4* in decidualizing cells. Thus, like other steroid hormone receptors, GR and MR modify decidual gene expression in a ligand-dependent and ligand-independent manner.

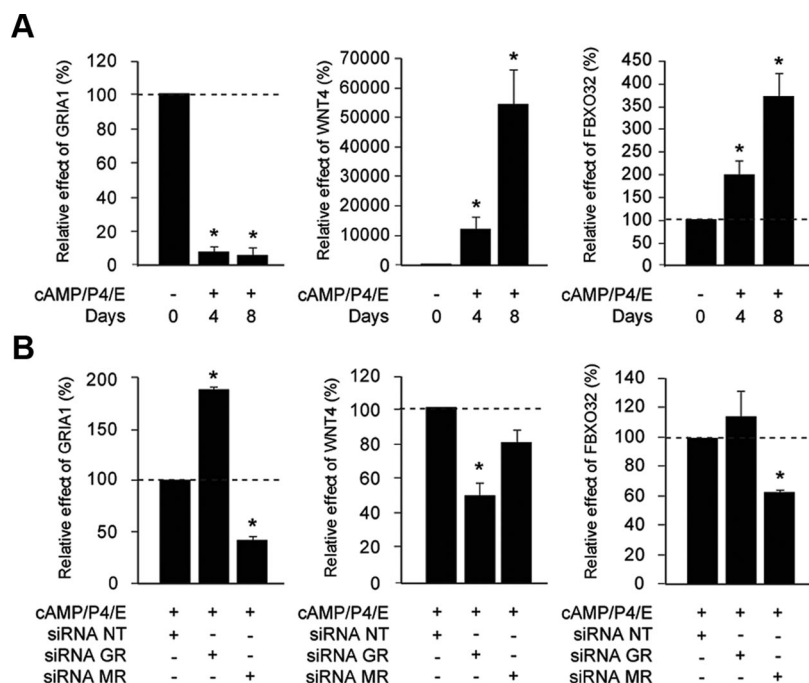
### GR limits the expression Krüppel-associated box domain containing ZNF (KRAB-ZNF) transcriptional repressors

After correction for multiple testing (Benjamini and Hochberg false discovery rate), gene ontology annotation revealed that the genes repressed by GR were strongly enriched for KRAB-ZNF proteins ( $P < 0.0001$ ). In fact, 18 members of this family of transcriptional repressors were up-regulated upon GR knockdown: ZNF91, ZNF92, ZNF100, ZNF253, ZNF254, ZNF311, ZNF420, ZNF484, ZNF486, ZNF585A, ZNF586, ZNF613, ZNF624, ZNF625, ZNF626, ZNF669, ZNF738, and ZNF791. Notably, the expression of five (ZNF181, ZNF223, ZNF254, ZNF625, and ZNF669) also increased upon MR knockdown (Supplemental Tables 4 and 5). Validation analyses demonstrated that the expression of *ZNF486* transcripts declines modestly upon treatment of HESCs with 8-bromo-cAMP, P4, and E (Fig. 5A, left panel). In agreement with the array findings, GR knockdown selective up-regulated *ZNF486* mRNA levels in differentiating HESCs by approximately 2-fold (Fig. 5A, right panel).

KRAB-ZNF proteins block transcriptional initiation by recruiting a variety of chromatin modifiers to promoters of target genes, resulting in an increase in H3K9me3 (26, 27). We therefore decided to monitor this repressive chromatin mark to determine whether GR plays a role in chromatin remodeling that underpins the decidual phenotype (28). Western blot analysis revealed a gradual decline in global H3K9me3 levels in response to differentiation cues (Fig. 5B, left panel). Knockdown of GR, and perhaps to a lesser extent of MR, was sufficient to reverse this response (Fig. 5B, right panel).

### MR is essential for retinoid metabolism and lipid droplet biogenesis

In contrast to GR, MR acts primarily as a transcriptional activator in decidualizing HESCs (Supplemental Tables 1 and 5). Perhaps the most striking observation is that several MR-induced genes encode for key enzymes involved in retinoid metabolism and cholesterol homeostasis, including retinol saturase, members of the short-chain DHRS (DHRS3, DHRS4, and DHRS4L2), the dehydrocholesterol reductase DHCR7, and the steroidogenic acute regulatory protein-related lipid transfer protein domain containing protein STARD5. To validate the array findings, we focused on DHRS3 (also known as retinal short-chain dehydrogenase/reductase 1). This enzyme was



**FIG. 4.** Expression and validation of putative GR- and MR-dependent genes in decidualizing HESCs. A, Expression of the putative GR- and MR-dependent genes in primary cultures treated 8-bromo-cAMP, P4, and E for the indicated time points. The results show mean fold change ( $\pm$ SEM) relative to levels in undifferentiated cells (dotted lines). B, Independent primary cultures ( $n = 3$ ) not used in the array analysis were first transfected with NT-, GR-, or MR-siRNA and subsequently treated with 8-bromo-cAMP, P4, and E for 4 d. Transcript levels were measured by RTQ-PCR and expressed as fold-induction ( $\pm$ SEM) relative to expression levels in transfected HESCs with NT-siRNA before differentiation (dotted lines). \*,  $P < 0.05$ .

highly induced at both mRNA and protein level in a time-dependent manner upon treatment of primary HESCs with 8-bromo-cAMP, P4, and E (Fig. 6A). MR knockdown attenuated the induction of *DHRS3* transcripts in decidualizing cells (Fig. 6B, right panel). To further explore MR dependency of *DHRS3* expression in decidualizing cells, primary cultures were treated with 8-bromo-cAMP, P4, and either aldosterone or RU26752, a MR antagonist (29, 30). Although addition of aldosterone potentiated the induction *DHRS3* in cells differentiated with 8-bromo-cAMP and P4, RU26752 completely abolished this response (Fig. 6B, right panel).

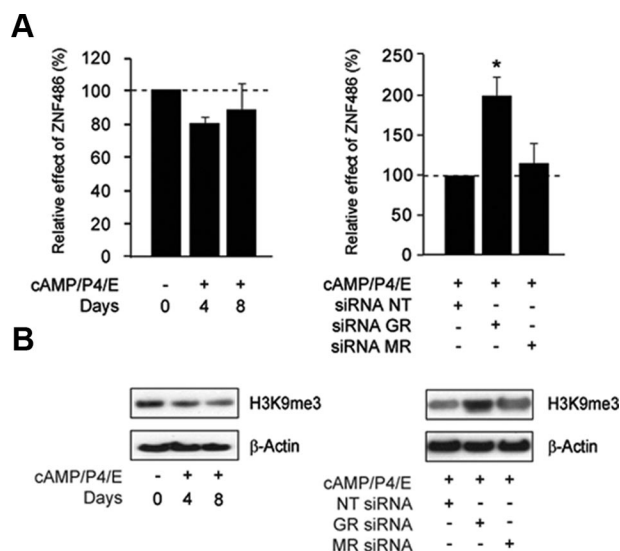
*DHRS3* has recently been implicated in endoplasmic reticulum (ER)-derived lipid droplet formation (31). Loading of primary HESCs with a cell-permeable lipophilic fluorescence dye, BODIPY 493/503, followed by confocal microscopy demonstrated that decidualization is associated with highly dynamic changes in the appearance of these cytoplasmic lipid droplets. Undifferentiated HESCs contain numerous small droplets. Unexpectedly, these lipid droplets were consistently less abundant after 4 d of differentiation (Fig. 6C). By d 8, the droplets had reaccumulated especially near the periphery of the cells. Four days later, fewer but larger and more centrally localized lipid droplets were present in decidualizing

HESCs (Fig. 6C). Next, we treated primary cultures with 8-bromo-cAMP, P4, and either aldosterone or RU26752 for 8 d. Interestingly, although addition of aldosterone favored the formation of larger droplets, RU26752 virtually abolished their presence all together (Fig. 6D).

## Discussion

Elevated circulating P4 levels maintain the maternal decidual response in pregnancy and are therefore indispensable for survival of the fetus. However, circulating levels of androgens, aldosterone, and F also increase in early pregnancy (10, 32–34). Furthermore, the endometrium expresses the full complement of cognate receptors as well as a host of steroidogenic enzymes that will determine the local bioavailability of different ligands. For example, increased expression accounts for the higher conversion of testosterone to dihydrotestosterone in secretory compared with proliferative endometrium (35, 36). We now show that P4 drives by *11 $\beta$ HSD1* expression, which is further reinforced by F production and signaling. This positive feedback mechanism renders *HSD11B1* one of the most highly induced genes upon HESC differentiation (18). We further show that decidualization is also associated with a rebalancing of corticosteroid receptors in favor of MR.

MR and GR show significant amino acid homology in their ligand- and DNA-binding domains with PR and androgen receptor (AR) (37). Together, these four receptors constitute the 3-ketosteroid receptor subfamily of nuclear receptors. The structural homology between these receptors implies that a degree of promiscuity may exist in the binding of various natural or synthetic ligands. This is indeed the case. For example, P4 binds MR, although the physiological consequences of this interaction, if any, are still unknown (38). Similarly, MPA, which unlike P4 does not have the propensity to partition onto glass and plastic in culture, is widely used to study PR responses *in vitro*, yet it is also a potent activator of GR and AR (35, 39). Structural homology also raises the possibility of functional redundancy between these receptors. In a previous study, we showed that this is not the case for PR and AR (35). By combining siRNA-mediated knockdown with ge-



**FIG. 5.** GR signaling plays a role in chromatin remodeling in decidualizing HESCs. A, ZNF486 transcript levels were measured in four primary cultures treated 8-bromo-cAMP, P4, and E for the indicated time points (*left panel*) as well as in cultures decidualized for 4 d after GR or MR knockdown (*right panel*). The results show mean fold change ( $\pm$ SEM) relative to levels in undifferentiated cells (*dotted lines*). \*,  $P < 0.05$ . B, Western blot analysis of H3K9me3 expression in total protein lysates from primary cultures treated 8-bromo-cAMP, P4, and E for the indicated time points (*left panel*) as well as from cultures decidualized for 4 d upon GR or MR knockdown (*right panel*).  $\beta$ -Actin served as a loading control.

nome-wide expression profiling, we showed that AR signaling is essential for cytoskeletal organization and cell cycle regulation in decidualizing cells. PR knockdown deregulated approximately nine times more genes than AR silencing (92 *vs.* 860 genes, respectively). Interestingly, a significant number of PR-regulated genes encode for membrane-bound receptors and intermediates in various signal transduction pathways, suggesting that P4 dependency of the decidual phenotype is, at least in part, accounted for by PR-dependent reprogramming of pathways activated by growth factors and cytokines (35).

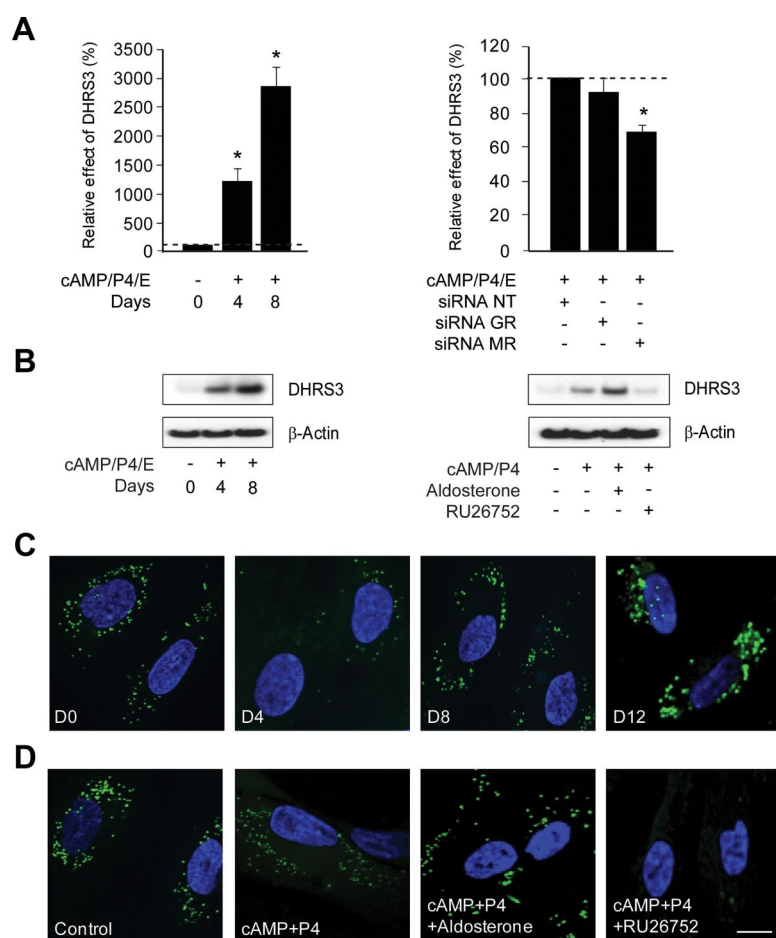
We adopted a similar approach to identify MR- and GR-dependent genes in differentiating HESCs. However, we decided against using MPA, because it introduces a bias in favor of GR responses. Instead, we combined 8-bromo-cAMP with P4 and added E as the substrate for 11 $\beta$ HSD1 conversion. After 4 d of treatment, we identified 179 and 107 deregulated genes upon GR or MR knockdown, respectively. Like PR (35), GR represses significantly more genes than it induces in decidualizing cells. The number of up- and down-regulated genes upon AR knockdown is comparable, whereas MR functions primarily to promote the expression of certain decidual genes. Cross-referencing of the array data identified only 12 genes that are regulated by PR or AR as well as GR or MR (Supplemental Table 6).

Mining of the GR-dependent genes yielded some unexpected results. For example, GR represses the expression of *SPP1* (osteopontin), a major component of the embryo-endometrial interactome (40), suggesting that glucocorticoid exposure during the window of implantation may interfere with embryo implantation. GR stimulated the induction of WNT4, a key component of P4 responses in both the uterus and breast (41, 42). Further, GR as well as MR signaling may be important for sustained cAMP activity in decidualizing cells by up-regulating the  $\alpha$ -catalytic subunit of protein kinase A (*PRKACA*) (Supplemental Table 5).

However, the most striking observation was that GR activity attenuates the induction of 18 KRAB-ZNF proteins. With 675 encoding genes, the C<sub>2</sub>H<sub>2</sub> zinc-finger proteins comprise the largest family of regulatory proteins in mammals, and 36% contain a KRAB domain (27, 43, 44). Over 50% of all human KRAB-ZFP genes are located in clusters on chromosome 19, including 15 of the 18 genes found to be regulated by GR in this study. The functions of these GR-repressed KRAB-ZFP genes are unknown with the exception of *ZNF420*, which encodes ATM and p53-associated krüppel type zinc finger protein, a negative regulator of p53-mediated apoptosis (45). However, it is well established that the KRAB domain confers a potent transcriptional repressor function by mediating specific interactions with a corepressor protein, krüppel-associated protein 1 (encoded by *TRIM28*), which in turn serves to recruit chromatin deacetylation machinery (46–48), as well as methyltransferase complexes (27). Because KRAB-ZNF transcription factors have been implicated in trimethylating H3K9 (27), we decided to monitor the cellular levels of this histone mark upon differentiation of HESCs. Perhaps somewhat fortuitously, this line of inquiry showed that decidualization is associated with a decline in global cellular H3K9me3 levels, which is disrupted upon GR knockdown.

Analysis of genes deregulated upon MR knockdown highlighted the dynamic changes in lipid droplet formation and retinoid metabolism that occur upon decidual transformation of HESCs. Although the MR dependency of these metabolic functions in decidualizing HESCs was unanticipated, it is in keeping with the observation that silencing of this nuclear receptor in murine adipocytes completely prevents lipid accumulation (49). In contrast, GR knockout only mildly impairs adipogenesis. Aldosterone has been shown to promote adipose conversion of 3T3-L1 and 3T3-F442A cells, whereas DEX inhibits terminal adipocyte maturation (50). Further, two MR-dependent genes in decidualizing HESCs, *RETSAT* and *DHRS3*, are strongly implicated in both intracellular lipid accumulation and retinoid metabolism (31, 51).





**FIG. 6.** MR-dependent induction of DHRS3 in decidualizing HESCs is associated with dynamic changes in cytoplasmic lipid droplets. DHRS3 expression was examined in parallel primary cultures treated with 8-bromo-cAMP, P4, and E for the indicated time points as well as after GR or MR siRNA knockdown by RTQ-PCR (A) or Western blot analysis (B). DHRS3 expression was analyzed in four independent primary cultures. The *left panel* shows percentage change ( $\pm$ SEM) in mRNA expression relative to levels in undifferentiated cells (*dotted lines*). C, Confocal micrographs showing lipid droplets stained with BODIPY (green) and nuclei stained with 4,6-diamidino-2-phenylindole (DAPI) (blue) in HESCs treated with 8-bromo-cAMP, P4, and E for the indicated time points. D, Representative confocal images of HESCs decidualized with 8-bromo-cAMP and P4 in the presence or absence of aldosterone or RU26752 for 8 d. Scale bar, 10  $\mu$ m.

Retinoic acid (RA), the biologically active metabolite of vitamin A (retinol), is essential for embryogenesis and maintenance of pregnancy. Both RA deficiency and excess cause severe fetal malformation, suggesting that retinoid metabolism must be tightly controlled at the fetomaternal interface (52–54). RA is derived from oxidation of all-*trans*-retinaldehyde (retinal), an unstable intermediate that fluxes between retinol and RA. Because DHRS3 is a retinaldehyde reductase that promotes storage of retinol in lipid droplets, its level of expression may be an important mechanism to modulate local RA availability (31, 55). We now show that DHRS3 levels are low in undifferentiated HESCs, but expression increases markedly upon decidualization, in parallel with the induction of 11 $\beta$ HSD1. In fact, both enzymes are structurally re-

lated members of the SDR superfamily that localize to the ER (56). The terminal enzymes involved in the synthesis of lipid droplets also localize to the ER and often to droplets themselves, as is the case for DHRS3 (31). Thus, the dynamic changes in the appearance and abundance of lipid droplets in decidualizing HESCs may at least partly reflect the changing nature of ER-resident enzymes. Lipid droplets are often viewed as mere energy storage facilities, containing predominantly neutral lipids and various proteins (57). Based on our observations, it is tempting to speculate that the constituents and functions of these lipid droplets also change upon decidualization of HESCs, perhaps becoming more akin to the retinyl ester storage particles (retinosomes) present in the eye (58).

In summary, our observations suggest that decidualization of the endometrium promotes the formation of a corticosteroid gradient at the fetomaternal interface. Although F is virtually absent in the placenta due to the abundant expression of 11 $\beta$ HSD2 (59, 60), its production on the maternal side may exert important autocrine as well as paracrine functions. For example, local F biosynthesis could constitute a major mechanism that protects the fetal allograft against a potential maternal immune response. Importantly, our data also suggest that MR is a central regulator of the metabolic functions of

the maternal decidua. This may be of particular importance to human pregnancy, because perfusion of the placenta is not established before 10–12 wk of gestation (61). Consequently, throughout the process of organogenesis (3–8 wk of pregnancy), fetal nutrition depends entirely on secretions produced by endometrial glands and decidualizing stroma (62, 63). The role of the endometrial 11 $\beta$ HSD1/GR/MR pathway in reproductive failure clearly warrants further investigation.

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